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Relative resistance of patient-derived envelope sequences to SERINC5-mediated restriction of HIV-1 infectivity

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ABSTRACT A host cellular transmembrane protein, SERINC5, inhibits HIV-1 infectivity when incorporated into progeny virions. Recent studies suggest that certain Envelope glycoproteins can resist SERINC5-mediated restriction. However, the underlying mechanism of Envelope glycoprotein-mediated resistance to SERINC5 restriction remains unclear. Here, we investigated the extent of sensitivity of patient-derived HIV-1 Envelope sequences to SERINC5-mediated restriction and examined Envelopes' characteristics in relation to SERINC5. A *nef*-deficient HIV-1 reporter was pseudotyped with Envelope sequences isolated from a total of 50 Tanzanians infected with non-B HIV-1 subtypes in the presence and absence of SERINC5 expression. The infectivity of resultant pseudoviruses was differentially reduced by SERINC5 by a median of 5.1-fold (IQR: 3.2–8.6) including five outliers showing ≥20-fold reduction, whereas the pseudovirus with the control NL4-3 envelope was reduced by 64-fold. The pseudovirus sensitivity to SERINC5-mediated restriction differed significantly among the subtypes of the envelope sequences but was not associated with any other Envelope characteristics or clinical parameters tested. Within some hosts, the pseudovirus sensitivity to SERINC5 varied substantially among Envelope sequences, with sensitive ones (as defined by ≥20-fold reduction) being underrepresented. Analysis of chimeric constructs between intra-host clones revealed that both N- and C-terminus of the Envelope sequences were responsible for SERINC5-mediated restriction. Taken together, these results demonstrate that the majority of naturally occurring Envelope sequences across multiple subtypes are relatively less sensitive to SERINC5-mediated restriction of infectivity and that naturally occurring polymorphisms at N- and C-terminal parts are important for this sensitivity.

IMPORTANCE Pathogenesis of HIV-1 is enhanced through several viral-encoded proteins that counteract a range of host restriction molecules. HIV-1 Nef counteracts the cell membrane protein SERINC5 by downregulating it from the cell surface, thereby enhancing virion infectivity. Some subtype B reference Envelope sequences have shown the ability to bypass SERINC5 infectivity restriction independent of Nef. However, it is not clear if and to what extent circulating HIV-1 strains can exhibit resistance to SERINC5 restriction. Using a panel of Envelope sequences isolated from 50 Tanzanians infected with non-B HIV-1 subtypes, we show that the lentiviral reporters pseudotyped with patient-derived Envelopes have reduced sensitivity to SERINC5 and that this sensitivity differed among viral subtypes. Moreover, we found that SERINC5 sensitivity within patient-derived Envelopes can be modulated by separate regions, highlighting the complexity of viral/host interactions.

KEYWORDS SERINC5, HIV-1, envelopes, patient-derived sequence, non-B subtypes

Host cells pose multiple barriers to the establishment of HIV-1 infection through a number of intrinsic factors that restrict the life cycle of HIV-1 at different stages,

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including the entry step mediated by viral Envelope proteins (1–5). Recently, Serine Incorporator Protein 5 (SERINC5), a member of multi-pass transmembrane proteins, has been shown to be incorporated into nascent virions of HIV-1 and other retroviruses and thereby restrict infection to target cells (6–9). In HIV-1, Nef protein counteracts SERINC5 restriction by interacting with the fourth intracellular loop of SERINC5 in conjunction with host molecules, downregulating it from cell membranes, and thereby preventing its incorporation into nascent virions (6, 8–17). Interestingly, this activity of patient-derived Nef sequences inversely correlated with plasma viral load (18, 19), suggesting the importance of SERINC5-mediated restriction of viral replication *in vivo*.

The mechanism by which virion-incorporated SERINC5 reduces virion infectivity is not clarified yet. Some studies suggested that SERINC5 may express its function by directly or indirectly interacting with other molecules including viral Envelope glycoproteins on the virion surface (7, 12). For instance, a single mutation at position 412 of SERINC5 located at the fifth extracellular loop to a non-aromatic amino acid resulted in reduced restriction function, without interfering with its incorporation to progeny virions (7). Also, SERINC5 has been found to have an impact on Envelope susceptibility to neutralizing antibodies (11, 12, 20, 21) and CCR5 antagonists (10), suggesting that SERINC5 may induce conformational changes in viral Envelope glycoproteins. The potential interaction between virion-incorporated SERINC5 and Envelope glycoproteins has become more plausible with the recent findings that Envelope sequences of certain HIV-1 reference strains, including JRFL, YU2, AD8, and BaL, are intrinsically less sensitive to SERINC5-mediated restriction even in the absence of Nef (6, 22–24). Whether this observation extends to patient-derived, non-B subtypes Envelope sequences is yet to be unveiled. In addition, motifs and regions that render HIV-1 Envelopes resistant to SERINC5 are not characterized. Because patient-derived Envelope sequences are highly diverse, it could be beneficial to understand the implication of this diversity in the context of SERINC5-mediated restriction, and its relationship to viral replication *in vivo*.

In this study, we examined the pseudovirus infectivity in the presence and absence of SERINC5 expression across a panel of patient-derived Envelope sequences isolated from treatment-naïve individuals infected with HIV-1 subtypes A1, C, D, and inter-subtype recombinant forms (isRFs). We also analyzed several characteristics of Envelope sequences for their association with sensitivity to SERINC5-mediated restriction. We found that the majority (>90%) of patient-derived Envelope sequences are relatively less sensitive to SERINC5-mediated restriction of infectivity. Using chimeras from intra-host Envelope clones exhibiting large differences in SERINC5 sensitivity, we show that regions in the N or C terminus of Envelope could modulate SERINC5 infectivity restriction.

RESULTS

Sensitivity of patient-derived Envelope sequences to SERINC5-mediated infectivity restriction

To assess the sensitivity of Envelope sequences to SERINC5-mediated restriction of HIV-1 infectivity in the absence of Nef expression, we first conducted a control experiment. A *nef*-deficient subtype B HIV-1 reporter vector, pSG3$_{\Delta ENV\Delta Nef}$-Luc2-IN/HiBit (25), was pseudotyped with Envelopes from subtype B reference strains, NL4-3, HXB2, BaL, JRFL, YU2, and AD8, in the presence and absence of SERINC5 expression. The production of progeny pseudoviral particles as expressed by p24 Gag levels was largely unaffected by SERINC5 expression ($P > 0.1$) (Fig. 1A). In contrast, infectivity of the resultant pseudoviruses with NL4-3 and HXB2 Envelopes was reduced by 64.6- and 72-fold, respectively, in the presence of SERINC5, whereas pseudovirus infectivity with other Envelopes (BaL, JRFL, YU2, and AD8) was reduced by a median of 4.9-fold (Fig. 1B). These results suggest that HIV-1 Envelopes of certain reference strains were intrinsically resistant to SERINC5, confirming the previous observations (6, 7, 10, 23).

Next, we assessed the sensitivity to SERINC5-mediated infectivity restriction of HIV-1 reporter viruses pseudotyped by a panel of 50 Envelope clones isolated from treatment-naïve individuals infected with HIV-1 in Tanzania. The patient-derived sequences formed

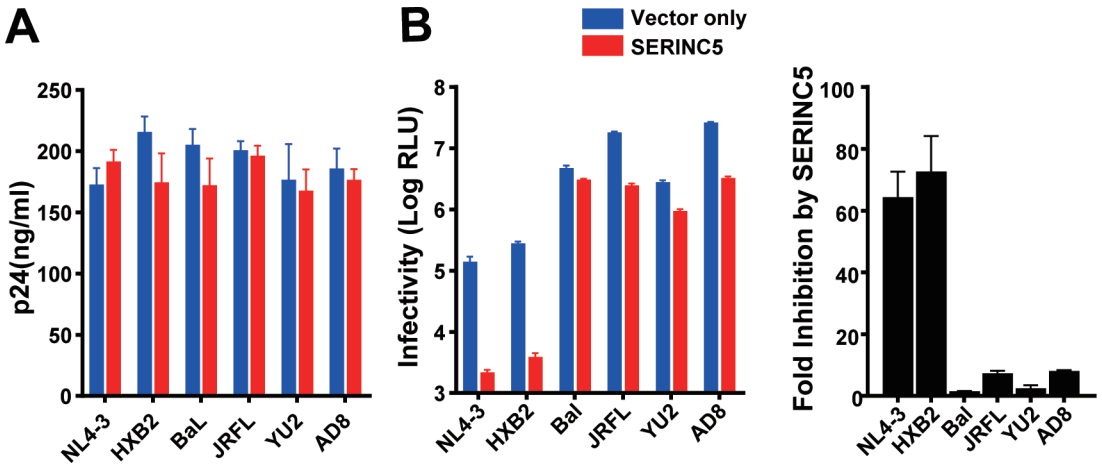

FIG 1 Differential effect of SERINC5 toward Envelopes of laboratory strains. Virion production (A) and infectivity (B) of the *nef*-deficient HIV-1 reporter vector pseudotyped with indicated Envelopes from reference strains in the presence and absence of SERINC5. HEK293T cells were co-transfected with *nef*-deficient HIV-1 reporter vector and *env*-encoding plasmids in the presence of empty vector or plasmid encoding SERINC5, and then virion production was quantified. The resultant pseudoviruses (3 ng of p24 antigen) were exposed to TZM-bl target cells, and the infectivity was determined. Fold reduction of infectivity by SERINC5 was calculated by the ratios of infectivity in the absence and presence of SERINC5. The data set shown is a representative set of two independent experiments. Data shown are the mean ± SD of triplicate determinations.

phylogenetically separated clusters accounted for sequences of subtypes A1, C, and D as well as some isRFs that were assigned between the clusters (Fig. 2A). Similar to the control experiment, the HIV-1 reporter was pseudotyped with these Envelopes in the presence and absence of SERINC5 expression. While the SERINC5 did not affect the production of progeny pseudoviral particles (Wilcoxon matched pairs, $P = 0.94$) (Fig. 2B), it significantly reduced the infectivity of the pseudoviruses (Wilcoxon-matched pairs, $P < 0.0001$) (Fig. 2C). When SERINC5-mediated infectivity restriction was obtained as the fold difference in infectivity in the presence and absence of SERINC5 expression, median infectivity reduction was 5.1-fold (IQR 3.2–8.6), with five outliers having the infectivity reduced by ≥20-fold (Fig. 2D).

Correlation between Envelope sensitivity to SERINC5 and clinical parameters of the participants

Next, we explored potential correlations between Envelope sensitivity to SERINC5 and clinical parameters of study participants. We did not find any significant correlation between plasma viral load and either the absolute infectivity of pseudoviruses or the fold inhibition of infectivity by SERINC5 (Spearman, all $P > 0.1$) (Table S1). In addition, a total of six participants were considered to have recent infections as assessed by a limiting antigen avidity enzyme immunoassay in the previous study (26). The Envelope sequences isolated from these individuals showed no significant differences in sensitivity to SERINC5 compared to Envelopes isolated from chronically infected patients (Kruskal–Wallis, $P = 0.81$). Of the Envelope clones isolated from the recent infection group, only one clone's infectivity (10a) was substantially reduced by SERINC5 (24.9-fold) (Table S1).

Envelope characteristics associating with sensitivity to SERINC5

We then asked whether the Envelope sensitivity to SERINC5 was different among HIV-1 subtypes. Infectivity of pseudoviruses prepared in the absence of SERINC5 showed no difference among the subtypes (Kruskal–Wallis, $P = 0.42$) (Fig. 3A). In contrast, Envelope sensitivity to SERINC5 as expressed by fold inhibition of infectivity showed significant difference among the subtypes (Kruskal–Wallis, $P = 0.008$). Specifically, the median infectivity inhibition by SERINC5 to subtype C Envelope clones [3.1-fold (IQR, 2.3–5.8)] was significantly lower than those of subtype A1 [7.3-fold (IQR, 5.3–11.03)] and subtype

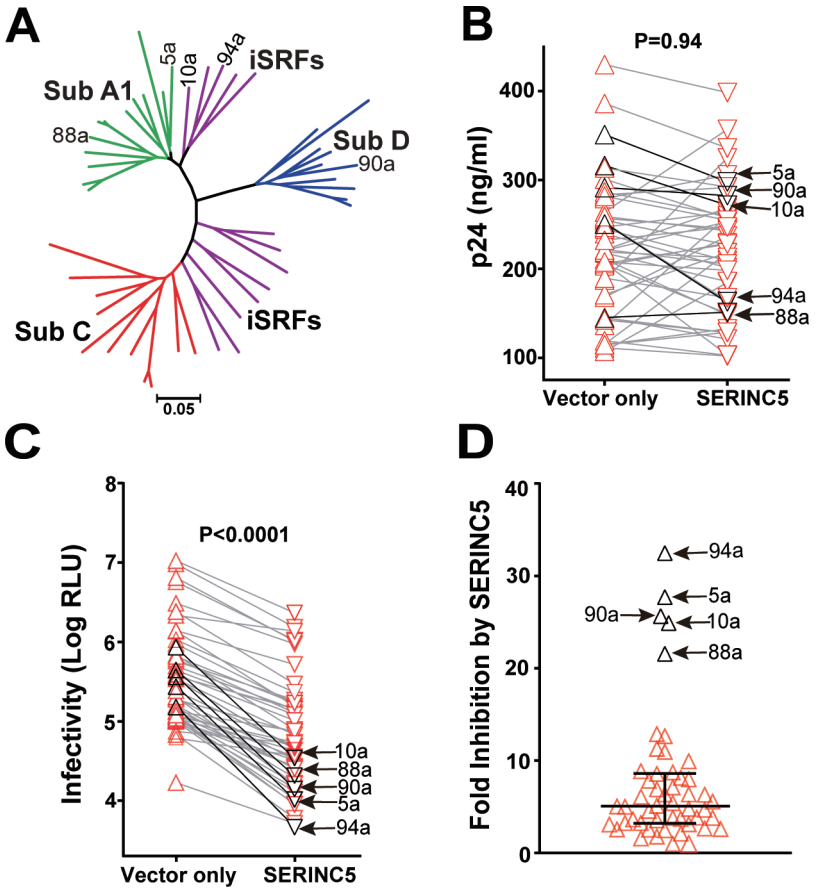

FIG 2 Sensitivity of patient-derived HIV-1 Envelope sequences to SERINC5. (A) Maximum likelihood phylogenetic tree of patient-derived Envelope clones (n = 50), colored according to subtypes: A1 (Green), C (Red), D (Blue), and isRFs (Purple). Virion production (B) and infectivity (C) of *nef*-deficient HIV-1 reporter vector pseudotyped with patient-derived Envelope clones in the presence and absence of SERINC5. The data set shown is a representative set of two independent experiments. Data shown are the mean ± SD of triplicate determinations. Statistical analysis was performed by a Wilcoxon matched-pairs test. (D) Fold reduction of infectivity by SERINC5. Symbols with black borders indicate clones with outliers (i.e., ≥20-fold reduction). Horizontal bars and whiskers indicate medians and interquartile ranges, respectively.

D [8.0-fold (IQR, 4.4–12.7)] (Mann–Whitney, $P \leq 0.01$) (Fig. 3B). Although isRFs were genetically highly diverse and did not form a unique phylogenetic cluster (Fig. 2A), the median infectivity inhibition by SERINC5 to a group of isRF Envelope clones [3.7-fold (IQR, 2.5–6.8)] was significantly lower than that of subtype A1 (Mann–Whitney, $P = 0.03$) (Fig. 3B). When the isRF group was stratified to three subgroups based on the recombination pairs (i.e., subtypes A1 and C, subtypes A1 and D, and subtypes C and D), no significant difference in sensitivity to SERINC5 was observed (Kruskal–Wallis, $P = 0.24$).

We also looked at whether intrinsic properties of the Envelope sequences could be associated with sensitivity to SERINC5. Co-receptor usage of patient-derived Envelope sequences used in this study has been phenotypically determined in the previous study (26) (Table S1). Envelope sensitivity to SERINC5 showed no difference between CCR5 and CXCR4-tropic Envelope sequences (Kruskal–Wallis, $P = 0.69$). In addition, Envelope loop properties as assessed by the number of potential glycosylation sites and number of amino acids in the V1/V2 hypervariable regions (Table S1) showed no association with sensitivity to SERINC5 (Spearman, all $P > 0.1$).

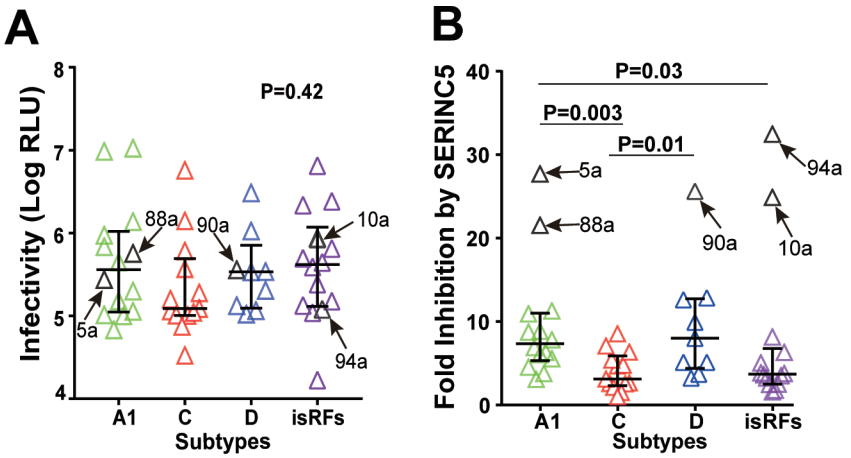

FIG 3 Effect of viral subtypes on sensitivity to SERINC5. Subtype difference in infectivity of pseudoviruses (A) and sensitivity to SERINC5 (B) of patient-derived Envelope sequences. The data set shown is a representative set of two independent experiments. Data shown are means ± SD of triplicate determinations. Horizontal bars and whiskers indicate medians and interquartile ranges, respectively. The statistical significance between all subtypes was calculated with the Kruskal–Wallis test, followed by the Mann–Whitney test.

Pseudovirus sensitivity to neutralizing antibodies and SERINC5

Next, we tested the pseudovirus sensitivity to a panel of broadly neutralizing monoclonal antibodies (mAbs) that target the gp41 membrane proximal external region (MPER); 4E10, 2F5, and 10E8, as well as the CD4-binding site (CD4bs); VRC01 and 3BNC117. Compared to the SERINC5-resistant Envelope sequences, the SERINC5-sensitive ones (the outliers shown in Fig. 2D) showed statistically significantly higher sensitivity to neutralization by the antibodies 4E10, 10E8, and 3BNC117 (Mann–Whitney, $P \leq 0.02$)

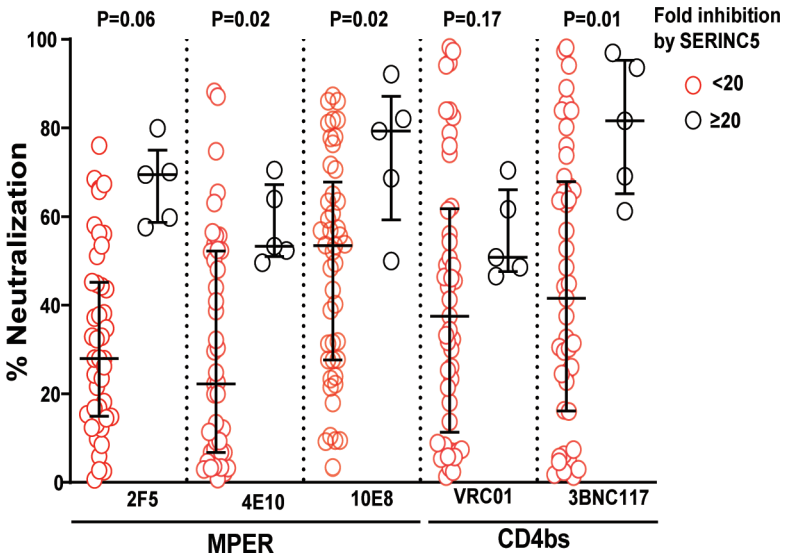

FIG 4 Neutralization sensitivity of Envelopes. Neutralization of patient-derived Envelopes against indicated mAbs at a concentration of 10 µg/mL. Envelopes were divided into two groups based on the sensitivity to SERINC5 as ≥ and <20-fold infectivity reduction. The data set shown is a representative set of two independent experiments. Data shown are means ± SD of duplicate determinations. The horizontal bars and whiskers represent the medians and interquartile ranges, respectively. Statistical significance was calculated by Mann–Whitney test.

and trended toward significance by antibody 2F5 (P = 0.06). Neutralization sensitivity to VRC01 was not significantly different between the Envelope groups (Fig. 4).

Differential sensitivity to SERINC5 within intra-host Envelope sequences

We wondered whether the sensitivity to SERINC5 may vary among pseudoviruses expressing different intra-host Envelope sequences due to the quasispecies nature of HIV-1. To address this, we attempted to isolate additional clones from five patients, NV05, NV10, NV88, NV90, and NV94, whose Envelopes showed relatively high sensitivity to SERINC5 (Fig. 2D). A total of 12 infectious clones were successfully isolated (Fig. 5); however, all three additional Envelope clones isolated from NV88 were genetically identical. From patient NV05, phylogenetically distinct clones were isolated and all showed lower inhibition by SERINC5 (less than ~12-fold) compared to the firstly isolated clone 5a (inhibition of 28-fold) (Fig. 5). Similarly, newly isolated clones, 10b and 10c from NV10; 90b, 90c, and 90d from NV90; 94b and 94c from NV94 showed the inhibitory sensitivities ranging from 5.3- to 16-fold; whereas the initially isolated clones, 10a, 90a, and 94a showed 24.9-, 25.6-, and 32.5-fold, respectively (Fig. 5). However, when tested for neutralization sensitivity, most of the intra-host clones showed comparable neutralizing sensitivity to 4E10 and 3BNC117 (Fig. 5).

Modulation of sensitivity to SERINC5 by distinct envelope regions

We capitalized on having largely genetically similar intra-host envelope clones that exhibited varying sensitivity to SERINC5 to identify the responsible regions or motifs. First, we chose Envelope clones 5a and 5c from NV05 to create chimeric clones by swapping their gp41 and gp120 regions (Fig. 6A). Analysis of sensitivity to SERINC5

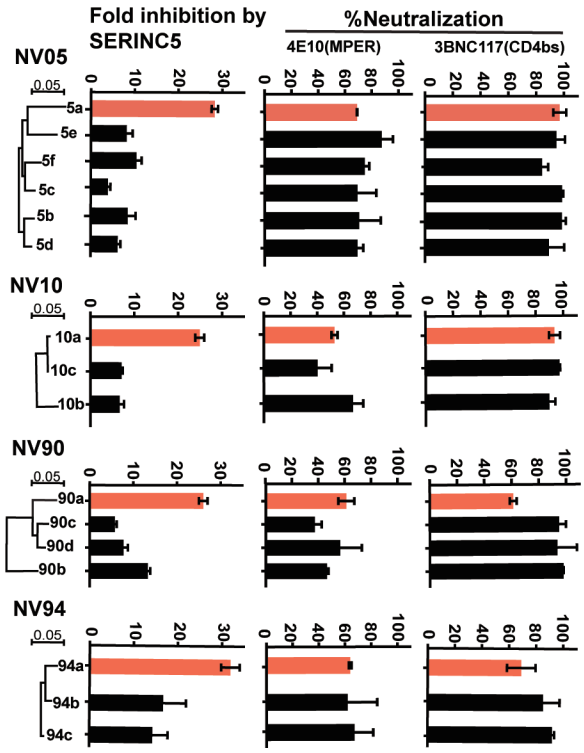

FIG 5 Pseudovirus sensitivity to SERINC5 in envelope sequences isolated from the same hosts Maximum likelihood phylogeny (*left*), sensitivity to SERINC5 (*middle*), and neutralizing sensitivity by the indicated mAbs (*right*) for intra-host clones from study participants NV05, NV10, NV90, and NV94. The data set shown is a representative set of two independent experiments. Data shown are means ± SD of triplicate determinations.

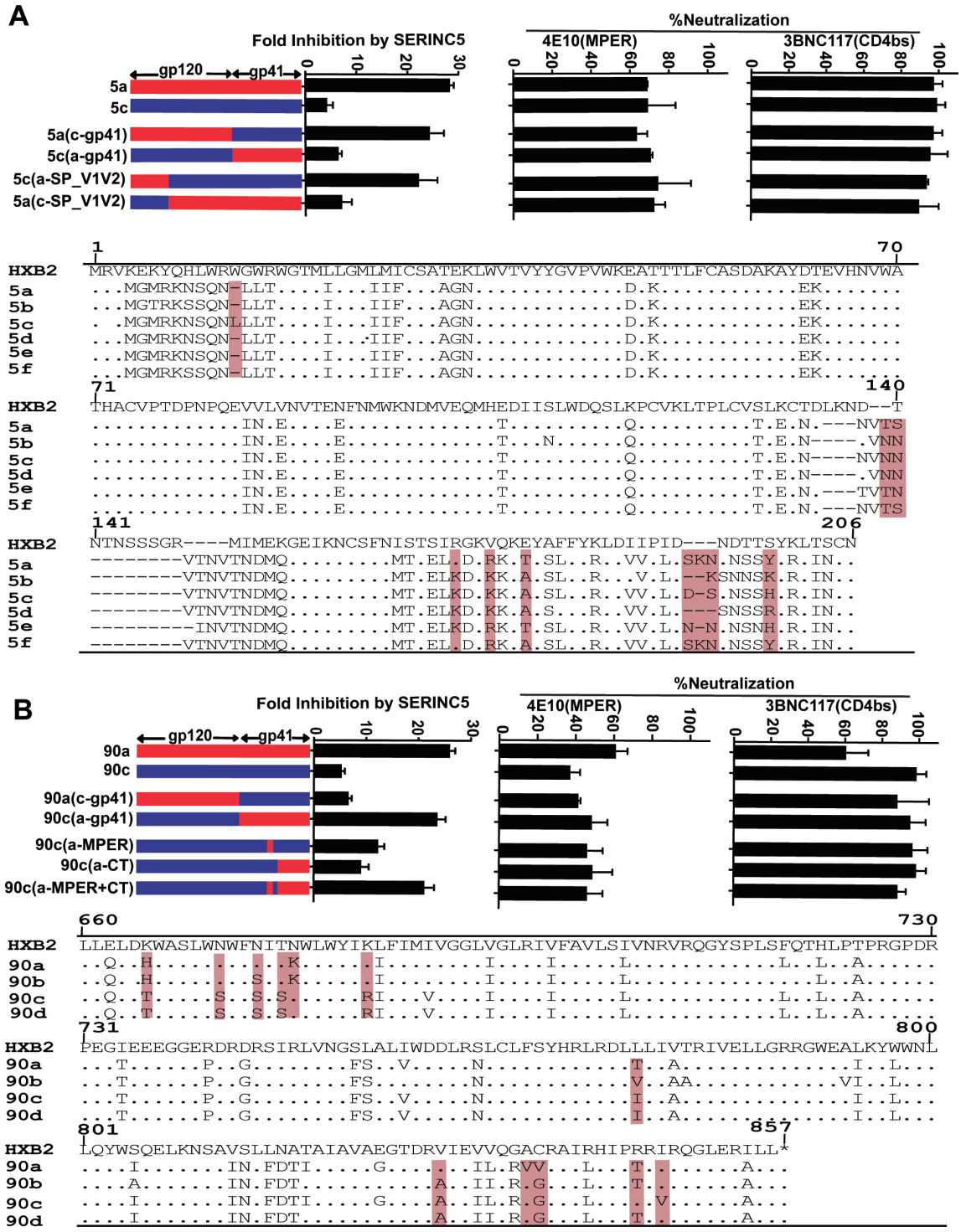

FIG 6 Pseudovirus sensitivity to SERINC5 and neutralization in chimeric envelope sequences. The sensitivity to SERINC5, and neutralization by mAbs, and the amino acid sequence alignments of the regions potentially responsible for SERINC5 sensitivity of the chimeric Envelope clones isolated from the patient NV05 (A) and NV90 (B). The data set shown is a representative set of two independent replicates. Data shown are means ± SD of triplicate determinations. Each sequence is aligned to HXB2 reference sequence, and amino acid residues showing differences between the clones are highlighted. Dots denote amino acid residues identical to HXB2, and dashes denote the absence of amino acids at those positions.

revealed that both chimeric clones, one having gp120 of 5a [designated 5a(c-gp41)] and the other with the gp120 of 5c [designated 5c(a-gp41)], largely retained sensitivity

of their parental clones to SERINC5 (Fig. 6A), suggesting that the motif responsible for SERINC5 sensitivity was within gp120. Analysis of chimeras with further breaking down the gp120 regions of 5a and 5c revealed that the N-terminal domain encompassing amino acid residues 1–206 (Signal peptide to Variable loop 2) was largely responsible for sensitivity to SERINC5 in this Envelope sequence (Fig. 6A). In contrast, when tested for neutralization sensitivity, these chimeric clones between 5a and 5c showed comparable neutralizing sensitivity to 4E10 and 3BNC117 (Fig. 6A). A total of 10 amino acid residues in this region were different between 5a and 5c, i.e., positions 13, 139, 140, 172, 175, 178, 192, 193, 194, and 199 (relative to HXB2 numbering) (Fig. 6A). Of these polymorphisms, four (at positions 139, 140, 175, and 178) were common to another clone 5d which also exhibited less sensitivity to SERINC5 (Fig. 6A).

In the same way, we constructed chimeric clones between 90a and 90c from NV90 by swapping their gp41 and gp120 regions. Analysis of sensitivity to SERINC5 revealed that the chimeric clone having gp41 of 90a [designated 90c(a-gp41)] gained the sensitivity; whereas the other chimeric clone having the gp41 of 90c [designated 90a(c-gp41)] exhibited decreased sensitivity (Fig. 6B). Construction of chimeras that further broke down the gp41 region of 90c(a-gp41) revealed that the MPER encompassing amino acid residues 660–683 and the C-terminal region encompassing amino acid residues 705–857 appeared to be responsible for sensitivity to SERINC5 in this Envelope sequence (Fig. 6B). In contrast, when tested for neutralization sensitivity, these chimeric clones between 90a and 90c showed comparable neutralizing sensitivity to 4E10 and 3BNC117 (Fig. 6B). A total of 13 amino acid residues were different between 90a and 90c including positions, 665, 671, 674, 676, 677, and 683 at MPER and also positions 775, 829, 836, 837, 845, and 847 at CT (Fig. 6B). Intriguingly, another clone (90b) also with relatively less SERINC5 sensitivity (Fig. 5) had similar amino acids with clone 90c at positions 665, 671, 674, 676, 677, and 683 at MPER, as well as 775, 836, and 837 at CT (Fig. 6B). However, further introduction of single substitutions at these sites could not lead to identifying particular residue(s) responsible for modulating SERINC5 sensitivity. Together, these data indicated that Envelope sensitivity to SERINC5 was influenced by naturally arising polymorphisms at distinct regions in the N and C terminus without substantially altering the neutralizing sensitivity.

DISCUSSION

In this study, we define the range of the sensitivity to SERINC5-mediated restriction of infectivity in the absence of Nef expression for naturally occurring Envelope variants isolated from plasma RNA of treatment-naïve individuals infected with multiple subtypes of HIV-1. We show that the majority of these variants (>90%) are relatively resistant to SERINC5. Furthermore, our analysis of intra-patient Envelope sequences highlights differential sensitivity to SERINC5 within quasispecies. These results suggest that naturally occurring Envelope sequences are adapted to SERINC5-mediated infectivity restriction during the chronic phase of HIV-1 infection *in vivo*.

The HIV-1 Envelope proteins are primary targets of neutralizing antibodies in addition to SERINC5. Previous studies (6, 9, 10, 23) have shown a correlation between the sensitivity of Envelopes to neutralizing antibodies and SERINC5. For instance, envelopes of neutralization tier 1 reference strains (i.e., NL4-3, HXB2, SF162, and 89.2) were sensitive to SERINC5-mediated restriction of infectivity, whereas those of tier 2/3 strains (i.e., AD8, YU2, and JRFL) were less sensitive to SERINC5. Another study (27) demonstrated that deletion of cytoplasmic tail region of NL4-3 Envelope reduced the sensitivity to both mAbs (notably 2F5 and 17b) and SERINC5. The observation in our study showing that SERINC5-sensitive Envelope clones were also sensitive to a panel of neutralizing mAbs (4E10, 10E8, and 3BNC117) appears to be in line with the previous findings. However, detailed analyses of chimeric Envelopes in this study revealed that SERINC5 sensitivity can be modulated by the distinct domains located at both N- and C-terminus of the Envelopes, while the sensitivity to mAbs remains largely unchanged among chimera constructs tested. These results suggest that sensitivity of naturally isolated Envelopes

to neutralization by mAbs and infectivity restriction by SERINC5 can be genetically separated without substantial functional tradeoffs between them. Further investigation is needed to unveil how the Envelope proteins can simultaneously adapt to host restriction factors and immune responses.

HIV-1 subtypes are not only defined by phylogenetic clusters, but also associated with functional differences. For instance, HIV-1 proteins encoded by patient-derived viral sequences such as Gag-Protease (28, 29), Envelope (26, 30, 31), Vif (32, 33), Vpu (34–36), Tat (37, 38), and Nef (36, 39, 40) were reported to display differential functionality between subtypes. In Tanzania, multiple major subtypes of group M HIV-1 make up the epidemic (26, 41–45), and in fact, patients infected with subtype D were associated with rapid disease progression than those with subtype A1 (46). In this study, subtype A1 Envelope sequences exhibited higher sensitivity to SERINC5-mediated infectivity restriction, compared to those of subtype C and isRFs. It would be intriguing to explore the clinical implications of the functional differences in Envelope sensitivity to SERINC5 among HIV-1 subtypes in future studies.

Viral genetic and functional studies of primary Envelope clones face numerous challenges and limitations. Although we investigated 62 of inter- and intra-host Envelope clones isolated from 50 individuals infected with major non-B subtypes of group M HIV-1, this panel does not capture the entirety of HIV-1 Envelope genetic diversity. We analyzed clinical implications using Envelope sequences isolated from one clone per patient, and the resultant association may be influenced by the choice of Envelope sequences; although for plasma viral load association with SERINC5 sensitivity, a separate analysis using median values of intra-host clones for NV05, NV10, NV90, and NV94 showed the same trend (Spearman correlation, $P = 0.21$). Also, as this study examined the cross-sectional cohort, it remains unclear whether reduced sensitivity of Envelope sequences to SERINC5 is maintained throughout the course of infection. Nevertheless, despite a substantial dynamic range of sensitivity of Envelopes to SERINC5, the vast majority of Envelope clones were relatively less sensitive to SERINC5 in the absence of Nef expression. While we used 293T cells to produce HIV-1 pseudoviruses with excellent consistency in infectivity, HIV-1 Envelope response to SERINC5 may be influenced by several factors including producer cells as well as the number of SERINC5 molecules, Envelope glycoproteins, and other molecules incorporated into progeny virions (22). In addition, we employed a *nef*-deficient proviral backbone to clarify the interaction between SERINC5 and Envelope clones; it remains unclear the extent to which the counteraction of SERINC5 by autologous Nef protein plays a role in viral replication. Despite these limitations, our study reveals relative resistance of patient-derived HIV-1 Envelopes to SERINC5-mediated infectivity restriction, and this reduced sensitivity is more pronounced in subtype C compared to other subtypes tested. In addition, we demonstrate that Envelope sensitivity to mAbs and SERINC5 is genetically separable and that two distinct regions at both ends of Envelope sequences play an important role in the sensitivity to SERINC5, indicating that the functional modulation of primary Envelope sequences is likely mediated by complex polymorphism networks.

MATERIALS AND METHODS

HIV-1 Envelope clones

Patient-derived Envelope clones used in this study were essentially reported in the previous study (26) (Table S1). In brief, DNA fragments encoding the *env* region were amplified by the nested PCR using plasma viral RNA isolated from consenting treatment-naïve HIV-1-infected participants [$N = 50$; median plasma viral load 4.8 log copies/mL (IQR 3.2–8.4)] in Tanzania, then cloned into a plasmid, and their genetic sequences were analyzed. The Genbank registration numbers are MZ147140–46, 147148, 147150–53, 147155, 147157, 147159–61, 147164, 147166–67, 147171–72, 147175, 147178, 147180, 147182–83, 147187, 147190–91, 147193, 147103, 147107, 147110, 147112–13, 147116–22, 147125–28, 147130–33, 147136–39, OR208770–OR208776. Maximum likelihood

phylogenetic tree and pairwise genetic distances between the entire region of these *envelope* sequences were calculated by MEGA (version 7.0) (47) using the Tamura-Nei model, with standard error estimates obtained by performing the Bootstraps procedure with 1,000 replicates, as previously described (48). Mutations were introduced into some of the Envelope clones by an overlap extension PCR as needed, and the mutated clones were confirmed by sequencing of a whole *env*-encoding region using Genetic Analyzer 3500xL (Applied Biosystems) as described previously (26).

Pseudovirus preparation

HIV-1 reporter virus ($pSG3_{\Delta ENV\Delta Nef}$-Luc2-IN/HiBit) (kindly provided by K. Tokunaga) (25) pseudotyped with the panel of patient-derived and reference Envelope sequences was prepared as previously described (7, 26). This HIV-1 reporter system (25) has HiBiT tag sequence (Promega) inserted at the 3′ end of the *pol* gene allowing to translate the integrase-HiBiT tag fusion protein followed by incorportion into the pseudovirus particles. In brief, HEK293T cells (American Type Culture Collection; 1.25×10^5/well) were seeded on a 24-well plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Sigma) and co-transfected with 500 ng of $pSG3_{\Delta ENV\Delta Nef}$-Luc2-IN/HiBit, 20 ng of an envelope clone, and 50 ng of pBJ5-SERINC5 (7) (kindly provided by H. Göttlinger) or the empty vector that had been pre-mixed with Lipofectamine 2000 (Thermo Fisher). Forty-eight hours later, culture supernatants containing psuedoviruses were harvested and clarified by centrifugation at 5,800 rpm for 3 min at 4°C. The viral titer of the pseudovirus preparations was then quantified based on HiBiT-associated chemiluminescence activity and normalized by the corresponding amount of p24 antigen as previously described (7, 26) .

Infectivity measurement

TZM-bl indicator cells (NIH AIDS Reagents Program; 1×10^4 cells per well) were seeded on a 96-well plate in DMEM supplemented with 10% fetal bovine serum and exposed to the pseudovirus inoculum (3 ng/mL) that had been prepared in the presence and absence of SERINC5 as above. Forty-eight hours later, the number of infected cells was assessed by firefly luciferase activity by using ONE-Glo Luciferase Assay system (Promega, USA). Relative luminescence unit was determined by subtracting the luminescence values in the presence of cells only.

Neutralization assays

The pseudovirus inoculum (3 ng/mL) that had been prepared in the absence of SERINC5 was pre-incubated with a panel of the following neutralizing mAbs: 4E10, 2F5, 10E8, 3BNC117, and VRC01 (NIH AIDS Reagent Program, United States) at 37°C for 1 h. Infectivity was then assessed as described above, and percent neutralization was calculated.

Statistical analysis

GraphPad Prism version 8 was used to perform statistical analyses described in the figure legends. Statistical significance was defined as a *P* value of less than 0.05.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (JVI00823-23-S0001.pdf). Patient clinical characteristics, properties of isolated Envelope sequences and their sensitivity to SERINC5 restriction.

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