Selective Disruption of SERINC5 Antagonism by Nef Impairs Simian Immunodeficiency Virus Replication in Primary CD4⁺ T Cells

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ABSTRACT The Nef proteins of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) enhance viral infectivity by preventing the incorporation of the multipass transmembrane protein serine incorporator 5 (SERINC5) and, to a lesser extent, SERINC3 into virions. In addition to counteracting SERINC5, SIV Nef also downmodulates several transmembrane proteins from the surface of virus-infected cells, including simian tetherin, CD4, and major histocompatibility complex class I (MHC I) molecules. From a systematic analysis of alanine substitutions throughout the SIV_{mac}239 Nef protein, we identified residues that are required to counteract SERINC5. This information was used to engineer an infectious molecular clone of SIV (SIV_{mac}239_{nef_{AV}}), which differs by 2 amino acids in the N-terminal domain of Nef that make the virus sensitive to SERINC5 while retaining other activities of Nef. SIV_{mac}239_{nef_{AV}} downmodulates CD3, CD4, MHC I, and simian tetherin but cannot counteract SERINC5. In primary rhesus macaque CD4⁺ T cells, SIV_{mac}239_{nef_{AV}} exhibits impaired infectivity and replication compared to wild-type SIV_{mac}239. These results demonstrate that SERINC5 antagonism can be separated from other Nef functions and reveal the impact of SERINC5 on lentiviral replication.

IMPORTANCE SERINC5, a multipass transmembrane protein, is incorporated into retroviral particles during assembly. This leads to a reduction of particle infectivity by inhibiting virus fusion with the target cell membrane. The Nef proteins of HIV-1 and SIV enhance viral infectivity by preventing the incorporation of SERINC5 into virions. However, the relevance of this restriction factor in viral replication has not been elucidated. Here, we report a systematic mapping of Nef residues required for SERINC5 antagonism. Counterscreens for three other functions of Nef identified two residues in the N-terminal domain of Nef that when mutated make the virus susceptible to SERINC5. Since Nef is multifunctional, separation of SERINC5 antagonism from its other functions allows comparison of the replication of viruses that are or are not sensitive to SERINC5. These experiments reveal the impact of SERINC5 on SIV replication in primary rhesus macaque CD4⁺ T cells.

KEYWORDS Nef, SERINC5, human immunodeficiency virus, infectivity, restriction factors, simian immunodeficiency virus

The five members of the serine incorporator (SERINC) family are multipass transmembrane proteins that share various degrees of sequence similarity (1). Of these proteins, SERINC5 and SERINC3 have been shown to inhibit retroviral replication (2, 3). In the absence of viral antagonists, retroviruses produced from SERINC-expressing cells incorporate these proteins as they bud from the plasma membrane (2, 3). The SERINC proteins packaged into virions can consequently inhibit viral fusion and prevent the delivery of viral contents to the target cell (2–5).

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Although it is known that SERINC5 impairs the fusion of viruses (2–5), the exact mechanism of virus restriction by SERINC5 remains poorly understood. Since SERINC family proteins potentially have a role in phospholipid metabolism, it has been speculated that lipid modifications caused by SERINC5 in human immunodeficiency virus (HIV) particles could restrict the virus (6–8). However, recent studies favor a model where SERINC5 physically interacts with the HIV envelope glycoprotein (Env) in a conformation-dependent manner (9, 10). This interaction appears to occur with the longest isoform of SERINC5 (1), which adopts a unique bipartite fold with 10 transmembrane helices (11). Specifically, residues in the fourth and fifth extracellular loops are required to inhibit viral infectivity (11–13). The result of these interactions is the inactivation of Env in the virus and the inhibition of fusion pore formation (4).

Restriction factors like SERINC5 are important components of innate immunity that potentially suppress viral replication. Successful viral pathogens therefore need to evolve mechanisms to counteract these restriction factors (14, 15). The resultant pressure for host survival in turn selects for amino acid changes in restriction factors. This can lead to the accumulation of amino acid differences in a restriction among different species. However, unlike restriction factors such as APOBEC3G/F, TRIM5 α , SAMHD1, and BST-2/tetherin, SERINC3 and SERINC5 do not display signs of positive selection (16–25). This suggests that the SERINC proteins are not evolving as rapidly as other restriction factors in response to the selective pressure of viral pathogens.

Nevertheless, multiple retroviruses have evolved mechanisms to counteract SERINC5 and enhance viral infectivity. The best-characterized example is the Nef protein of primate lentiviruses, which removes SERINC5 from sites of virus assembly by AP-2-dependent endocytosis (2, 3, 26). In a similar fashion, the S2 protein of equine infectious anemia virus (EIAV) (27) and Glycogag of murine leukemia virus (MLV) (28, 29) also enhance infectivity by counteracting SERINC5. In addition to antagonism by Nef, the Env proteins of some HIV-1 isolates are resistant to SERINC5 (30). The evolution of diverse mechanisms to overcome restriction by SERINC5 suggests that it represents an important barrier to virus replication.

In addition to counteracting SERINC5, the Nef proteins of primate lentiviruses perform other functions that promote viral replication and pathogenesis (31–33). Simian immunodeficiency virus (SIV) Nef counteracts simian tetherin to facilitate virus release from infected cells (34, 35). HIV-1 and SIV Nef proteins also downregulate CD4 (36, 37) and major histocompatibility complex class I (MHC I) molecules (38) from the cell surface. CD4 downregulation prevents the exposure of CD4-induced epitopes in Env, which protects virus-infected cells from antibody-dependent cellular cytotoxicity (39), and Nef-mediated downmodulation of MHC class I molecules from the cell surface protects virus-infected cells from CD8⁺ T cell responses (40, 41). CD4⁺ T cell activation is also impaired by downmodulation of the T cell receptor (TCR)-CD3 complex by Nef (42, 43). Since Nef is a multifunctional protein, the relative importance of individual Nef functions for lentiviral replication is not well understood.

In this study, we sought to understand the functional relevance of SERINC5 counteraction by SIV Nef. Since Nef is the viral gene product of SIV that counteracts SERINC5, a library of 132 mutants with pairwise substitutions throughout Nef were screened to identify amino acid changes that disrupt SERINC5 antagonism without affecting other functions. These experiments demonstrate that the anti-SERINC5 activity of Nef is separable from other functions. This information was used to engineer an infectious molecular clone of SIV that is selectively susceptible to SERINC5. By comparing the replication of viruses that are or are not susceptible to SERINC5, we demonstrate the impact of SERINC5 on lentiviral replication.

RESULTS

Impairment of SIV infectivity by SERINC3 and SERINC5. The effects of SERINC3 and SERINC5 on SIV infectivity were assessed by producing virus in Jurkat T antigen (JTAG) cells with or without knockout mutations in *SERINC3*, *SERINC5*, or both.

FIG 1 SERINC5 inhibits SIV infectivity and is antagonized by Nef. Wild-type JTAG cells or JTAG cells with knockout mutations in *SERINC3* and/or *SERINC5* were cotransfected with SIV_{mac239Δnef} proviral DNA and constructs expressing wild-type (WT) Nef or Nef with a G2A substitution that prevents myristoylation. The infectivity of the viruses released into the supernatant was measured on C8166-SEAP cells. The infectivity of the viruses produced in the presence of wild-type Nef, Nef G2A, or an empty vector is shown. Error bars indicate the standard deviations of the means from three independent experiments. Differences in infectivity were compared using two-tailed Student's *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

Although *nef*-deleted SIV produced in parental JTAG cells was poorly infectious, *trans*-complementation with wild-type Nef rescued virus infectivity. In contrast, *trans*-complementation with a Nef mutant with a glycine-to-alanine substitution at position 2 (G2A), which broadly impairs Nef function by preventing protein myristoylation and association with cellular membranes, did not restore viral infectivity (Fig. 1). Similar to viruses from parental JTAG cells, viruses produced in SERINC5⁺ (SERINC3 knockout [S3KO]) JTAG cells showed enhanced infectivity in the presence of Nef. However, viruses produced from SERINC3⁺ (S5KO) JTAG cells and SERINC3⁻ SERINC5⁻ (double knockout [DKO]) JTAG cells showed minor or no differences in infectivity (Fig. 1). These observations confirm that SERINC5, and to a lesser extent SERINC3, restrict *nef*-deleted SIV in JTAG cells. Since cells expressing SERINC5 recapitulate the Nef-dependent enhancement of viral infectivity, S3KO cells were used to screen Nef mutants for SERINC5 antagonism.

Separation of SERINC5 antagonism from other functions of Nef. To define amino acids in SIV Nef that contribute to SERINC5 antagonism, 132 pairwise alanine and valine substitutions spanning all 263 amino acids of Nef were screened for the ability to rescue the infectivity of *nef*-deleted SIV_{mac239} in S3KO cells. Fifty-four of these Nef mutants failed to restore the infectivity of SIV_{mac239Δnef} (Fig. 2). The infectivity of virus produced in the presence of these Nef mutants was within 3 standard deviations of that of virus produced in the presence of the nonmyristoylated Nef G2A control (Fig. 2B to D). These experiments identified 13 substitutions in the N-terminal domain (Fig. 2B), 28 substitutions in the globular core (Fig. 2C), 6 substitutions in the flexible loop (Fig. 2D), and 7 substitutions in the C-terminal domain (Fig. 2D) that disrupt SERINC5 antagonism.

To identify substitutions that selectively impair SERINC5 antagonism, the library of mutants was also screened for their ability to downmodulate CD4 and MHC I molecules, which require distinct protein sequences and occur by AP-2- versus AP-1-dependent endocytosis (36, 37, 44). CD4 downregulation assays were performed by transfecting TZM-bl cells and MHC I downregulation assays were performed by transfecting 293T cells with bicistronic vectors that express Nef with green fluorescent protein (GFP). By comparing the mean fluorescence intensity (MFI) of staining for CD4 and MHC I on the surface of GFP-positive (GFP⁺) cells to that on cells transfected with a control empty vector, these functions of Nef were quantified. Loss of function was defined as expression levels of CD4 or MHC I of >3 standard deviations above those in cells transfected with wild-type Nef (Fig. 3 and 4, dotted lines). Of the 132 Nef mutants

FIG 2 Identification of residues required for SERINC5 antagonism. (A) Predicted amino acid sequence of *SIV_{mac}239* Nef. The sequences are color-coded to represent the N-terminal domain (green), the globular core domain (blue), the flexible loop domain (red), and the C-terminal domain (pink). (B to D) SERINC5⁺ S3KO JTAG cells were cotransfected with *SIV_{mac}239Δnef* proviral DNA and constructs expressing either wild-type Nef, Nef G2A, or the indicated Nef mutants. The infectivity of virus released into the supernatant was measured on C8166-SEAP cells or TZM-bl cells and expressed relative to virus produced in the presence of wild-type Nef. Filled circles represent data from independent experiments for Nef mutants with substitutions in the N-terminal domain (green) (B), the globular core domain (blue) (C), and the flexible loop (red) and the C-terminal domain (pink) (D). Bars indicate average infectivities for each mutant, and error bars indicate standard deviations of the means from multiple independent experiments. The dotted lines indicate 3 standard deviations over the mean infectivity of virus *trans*-complemented with the Nef G2A mutant.

tested, 35 mutants were deficient for CD4 downmodulation (Fig. 3A to C), and 42 mutants were deficient for MHC I downmodulation (Fig. 4A to C).

Steady-state levels of protein expression were also assessed by Western blot analysis. Of the 54 Nef mutants deficient for SERINC5 antagonism, 24 exhibited a marked

FIG 3 Identification of residues required for CD4 downmodulation. Surface expression levels of CD4 were compared on TZM-bl cells transfected with bicistronic vectors expressing wild-type Nef or the indicated Nef mutants together with GFP. The mean fluorescence intensity of CD4 staining on GFP⁺ cells was calculated relative to cells transfected with an empty vector. Filled circles represent data from individual experiments for Nef mutants with substitutions in the N-terminal domain (green) (A), the globular core domain (blue) (B), and the flexible loop (red) and the C-terminal domain (pink) (C). Bars indicate average CD4 levels in the presence of each mutant, and error bars represent the standard deviations of the means from multiple independent experiments. The dotted line indicates 3 standard deviations over mean CD4 staining on cells expressing wild-type Nef.

decrease in expression indicative of defects in protein folding and/or stability and were not analyzed further (Fig. 5). Among the remaining Nef mutants with appreciable levels of protein expression, eight were previously shown to retain the ability to antagonize tetherin (45) and were shown in our current analysis to also downregulate CD4 (Fig. 6B and Table 1) and MHC I (Fig. 6C and Table 1). These include Nef mutants with substitutions at positions 31 and 32, 52 and 53, 56 and 57, 72 and 73, 90 and 91, 100 and 101, 102 and 103, and 132 and 133 (Fig. 6 and Table 1).

To determine which residues among these double substitutions contribute to

FIG 4 Identification of residues required for MHC class I downmodulation. Surface expression levels of MHC class I were compared on 293T cells transfected with a bicistronic vector expressing wild-type Nef or the indicated Nef mutants together with GFP. The mean fluorescence intensity of MHC class I staining on GFP⁺ cells was calculated relative to that on cells transfected with an empty vector. Filled circles represent data from individual experiments for mutant Nef proteins with substitutions in the N-terminal domain (green) (A), the globular core domain (blue) (B), and the flexible loop (red) and the C-terminal domain (pink) (C). Bars indicate average MHC I levels for each mutant, and the error bars represent the standard deviations of the means from multiple independent experiments. The dotted line indicates 3 standard deviations over mean MHC I expression on cells expressing wild-type Nef.

SERINC5 antagonism, additional Nef mutants with single-amino-acid substitutions were constructed. These Nef mutants were tested for their ability to rescue the infectivity of SIV_{mac}239Δnef produced from S3KO cells, downmodulate CD4 and MHC I molecules, and facilitate virus release in the presence of rhesus macaque tetherin. Five of these mutants partially impaired anti-SERINC5 activity similar to the G2A mutant (Fig. 7A). These include alanine substitutions for L31, N72, P73, D91, and Y133 (Fig. 7A). With the exception of Y133A, which partially impairs CD4 downmodulation, these Nef

FIG 5 Steady-state expression of Nef mutants deficient for SERINC5 antagonism. Steady-state expression levels for each of the Nef mutants were compared to that of wild-type Nef by Western blot analysis of transfected 293T cells. Blots were probed with a monoclonal antibody to Hsp90 as a loading control and with polyclonal serum from SIV-infected macaques to detect Nef. Steady-state levels of Nef in cell lysates were estimated from the band intensities of each of the Nef mutants relative to wild-type Nef.

mutants were able to downmodulate CD4 (Fig. 7B) and MHC I (Fig. 7C). Additionally, these Nef mutants also facilitate virus release in the presence of tetherin (Fig. 7D). The results of the virus release assay were corroborated by Western blot analyses comparing p55 Gag expression in cell lysates to the accumulation of p27 capsid in the cell culture supernatant (Fig. 7E).

Combinations of D91A or L31A with N72A and P73A were also tested for anti-SERINC5 activity. Although the triple-alanine substitutions were defective for SERINC5 antagonism, the infectivity of *SIV_{mac}239Δnef trans*-complemented with the N72A P73A double mutant was the most defective and did not differ detectably from virus *trans*-complemented with the Nef G2A mutant (Fig. 7F).

FIG 6 Identification of residues in SIV Nef that contribute to SERINC5 antagonism. The relative infectivity of Nef trans-complemented *SIV_{mac}239Δnef* (A), the relative levels of CD4 (B) and MHC class I (C) surface expression, and steady-state levels of Nef (D) in transfected cells are shown for the indicated Nef mutants. Infectivity was measured as described in the legend of Fig. 2. CD4 and MHC I expression levels were determined on TZM-bl and 293T cells, respectively, as described in the legends of Fig. 3 and 4. Steady-state levels of Nef expression were compared as described in the legend of Fig. 5. Error bars indicate standard deviations of the means from at least three independent experiments. Differences in Nef phenotypes were compared using two-tailed Student's *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

An infectious molecular clone of SIV with substitutions in Nef that impair SERINC5 antagonism. To create an infectious molecular clone of SIV with a combination of mutations that would be difficult to revert to wild-type, codon changes were selected to require at least 2 nucleotide substitutions at each position to restore the wild-type residue. Since the P73A substitution is encoded by a single nucleotide change, a P73V substitution was introduced using a codon that differs by 2 nucleotides from the wild-type sequence. These mutations were combined with the N72A substitution, which is also encoded by 2 nucleotide differences from the wild-type codon. The infectivity of this virus (*SIV_{mac}239nef_{AV}*) was greatly impaired compared to wild-type *SIV_{mac}239*, but not quite to the same extent as the outright deletion of *nef*, as reflected by some residual infectivity relative to *SIV_{mac}239Δnef* (Fig. 8A). Western blot analysis of viruses produced in 293T cells showed no differences in virion production or Env incorporation for *SIV_{mac}239nef_{AV}* in comparison to *SIV_{mac}239* and *SIV_{mac}239Δnef* (Fig. 8B), suggesting that the loss of infectivity is not due to defects in the virions produced. The loss of infectivity can, however, be attributed to the incorporation of SERINC5 into virions. This was confirmed by the detection of human and rhesus macaque SERINC5 in *SIV_{mac}239Δnef* and *SIV_{mac}239nef_{AV}* particles, but negligible SERINC5 in wild-type *SIV_{mac}239* particles (Fig. 8B). Consistent with differences in viral infectivity, SERINC5 downmodulation from the surface of cells transfected with *SIV_{mac}239nef_{AV}* was impaired relative to that in cells transfected with wild-type *SIV_{mac}239*, although not quite to the same extent as in cells transfected with *SIV_{mac}239Δnef* (Fig. 8C). Furthermore, in accordance with the absence of species specificity in SERINC5 antagonism by Nef (21, 22), differences in the downmodulation of human and rhesus SERINC5

TABLE 1 Summary of the properties of the SIV Nef mutants selectively defective for anti-SERINC5 activity^a

	% SERINC5 Antagonism ^a	% CD4 Expression ^b	% MHC I Expression ^c	Nef Levels ^d
Nef	100.0%	45.4%	37.7%	1.00
G2A	9.8%	74.6%	74.9%	1.18
31-32	5.9%	45.2%	30.7%	1.24
52-53	16.4%	47.6%	40.4%	1.04
56-57	21.7%	50.9%	39.5%	0.53
72-73	23.2%	59.2%	46.8%	0.86
90-91	21.3%	57.1%	45.2%	1.05
100-101	22.6%	47.7%	45.6%	0.84
102-103	16.0%	46.3%	54.0%	0.96
132-133	19.4%	68.4%	49.6%	0.63

^aThe mutants deficient for SERINC5 antagonism are indicated in different colors corresponding to their respective domains of Nef, including the N-terminal domain (green) and the globular core domain (blue). ^a, JTAG S3KO cells were cotransfected with SIV_{mac239}Δnef proviral DNA and the indicated Nef expression constructs. The infectivities of the viruses produced were measured on C8166-SEAP cells or TZM-bl cells. The infectivities of viruses *trans*-complemented with the indicated Nef mutants were normalized to those of viruses *trans*-complemented with wild-type Nef. ^b, CD4 levels were calculated as a percentage of CD4 staining (MFI) on cells transfected with each of the indicated Nef expression constructs relative to CD4 staining on cells transfected with an empty vector. ^c, MHC class I levels were calculated as a percentage of MHC I staining (MFI) on cells transfected with each of the indicated Nef expression constructs relative to MHC I staining on cells transfected with an empty vector. ^d, Steady-state levels of Nef in cell lysates were estimated by calculating the band intensity of each of the Nef mutant proteins and normalized to wild-type Nef.

were similar (Fig. 8C). To ensure that SIV_{mac239}nef_{AV} was also able to antagonize tetherin, virus release in the presence of increasing amounts of rhesus tetherin was measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 8D) and corroborated by Western blot analysis (Fig. 8E). Whereas virus release for SIV_{mac239}Δnef was impaired with increasing expression of rhesus tetherin, the levels of virus release for SIV_{mac239}nef_{AV} and SIV_{mac239} were nearly indistinguishable. SIV_{mac239}nef_{AV} is therefore susceptible to inhibition by SERINC5 while retaining wild-type resistance to tetherin.

Phenotypic characterization of SIV_{mac239}nef_{AV} in primary lymphocytes. The phenotype of SIV_{mac239}nef_{AV} was verified by comparing Nef functions to those of wild-type SIV_{mac239} and SIV_{mac239}Δnef in primary CD4⁺ lymphocytes from six rhesus macaques. Although the infectivity of SIV_{mac239}nef_{AV} produced in primary CD4⁺ T cells was slightly higher than that of SIV_{mac239}Δnef, this virus exhibited a nearly complete loss of infectivity relative to wild-type SIV_{mac239} (Fig. 9A and F). In contrast, SIV_{mac239}nef_{AV} retained the ability to downmodulate CD4 from the surface of infected cells, albeit with a modest but significant impairment relative to SIV_{mac239} (Fig. 9B and G). As for other functions of Nef, SIV_{mac239}nef_{AV} retained the ability to downmodulate CD3 (Fig. 9C and H), MHC I (Fig. 9D and I), and tetherin (Fig. 9E and J) to levels that did not differ detectably from those in cells infected with SIV_{mac239}. Thus, SIV_{mac239}nef_{AV} is selectively deficient for SERINC5 antagonism while maintaining other AP-1- and AP-2-dependent functions of Nef.

Replication of SIV_{mac239}nef_{AV} is impaired in SERINC5-expressing cell lines and primary rhesus macaque lymphocytes. Virus replication was assessed in cell lines that do and do not express appreciable levels of SERINC5 (2, 28, 46). Wild-type SIV_{mac239}, SIV_{mac239}Δnef, and SIV_{mac239}nef_{AV} replicate to similar levels in CEMx174 cells (Fig. 10A), where SERINC5 expression is negligible (2, 28). However, the replication of SIV_{mac239}nef_{AV} and SIV_{mac239}Δnef was delayed relative to SIV_{mac239} in SupT1-CCR5 cells (Fig. 10B), which express SERINC5 (2, 28). To confirm the phenotype of SIV_{mac239}nef_{AV} under more physiological conditions, virus replication was also compared in primary rhesus macaque CD4⁺ lymphocytes (Fig. 10C to E). Activated peripheral blood mononuclear cells (PBMCs) from three rhesus macaques were depleted of CD8⁺ lymphocytes and infected with SIV_{mac239}, SIV_{mac239}Δnef, and SIV_{mac239}nef_{AV}. Although the replication of viruses in primary CD4⁺ T cells from different animals was variable, SIV_{mac239}Δnef and SIV_{mac239}nef_{AV} replicated with similar kinetics and

FIG 7 Residues in SIV Nef that contribute to antagonism of SERINC5. Differences in viral infectivity (A), CD4 downmodulation (B), MHC I (C) downmodulation, tetherin antagonism (D), and protein expression (E) are shown for the indicated Nef mutants. (F) Differences in the infectivities of *SIV_{mac}239Δnef* trans-complemented with the indicated combinations of mutations in Nef are also shown. Infectivity was measured as described in the legend of Fig. 2, CD4 and MHC I expression levels were measured as described in the legends of Fig. 3 and 4, and steady-state levels of Nef expression were compared as described in the legend of Fig. 5. (D) Tetherin antagonism was assessed by measuring the accumulation of SIV p27 in the supernatant of 293T cells cotransfected with an *SIV_{mac}239Δnef* clone, constructs expressing the indicated Nef mutants, and an expression construct for rhesus macaque tetherin. Percent maximal virus release was calculated relative to virus release in control transfections without tetherin. (E) Differences in tetherin antagonism were confirmed by Western blot analysis of transfected cell lysates and virus recovered from the cell culture supernatant. (F) Differences in Nef phenotypes were compared using two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; "ns," not significant).

displayed impaired growth compared to wild-type *SIV_{mac}239* (Fig. 10C to E). Thus, disruption of SERINC5 antagonism impairs SIV replication in primary CD4⁺ lymphocytes.

DISCUSSION

The incorporation of SERINC5 into virus particles restricts lentiviral infectivity by impairing fusion with the target cells (2, 3, 5, 47). The Nef protein expressed by primate

FIG 8 Characterization of a SERINC5-sensitive SIV Nef mutant. (A) SIV_{mac}239, SIV_{mac}239Δnef, and SIV_{mac}239nef_{AV} were produced by transfection of JTAG S3KO cells, and the infectivities of these viruses on TZM-bl cells were compared to that of wild-type SIV_{mac}239. (B) Parental 293T cells and 293T cells expressing rhesus or human SERINC5 with an HA tag (SERINC5-iHA) were transfected with plasmid clones for each virus. Immunoblots of cell lysates and virions were probed with antibodies to the viral Gag (p55 and p27), Nef, and gp41 proteins and to the HA tag (S5-iHA) and Hsp90 as a loading control. (C) Differences in the surface expression of human and rhesus SERINC5-iHA on transfected cells are shown as histograms for one representative experiment and as bar graphs for average values from three independent experiments. (D) The accumulation of SIV p27 in the cell culture supernatant of 293T cells transfected with plasmid clones for the indicated viruses and increasing amounts of a construct expressing rhesus tetherin was measured by a p27 antigen capture ELISA. Percent maximal release was calculated relative to control transfections in the absence of tetherin. (E) Differences in virus release were corroborated by Western blot analysis of virions and cell lysates. In panels A and C, Error bars indicate standard deviations of the means from three independent experiments, and differences were compared using two-tailed Student's *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

lentiviruses overcomes this restriction by removing SERINC5 from sites of virus assembly by AP-2-dependent endocytosis (2, 3, 26, 48). In this study, we comprehensively define residues throughout the Nef protein that are required to antagonize SERINC5. Although the amino acid sequences required to counteract SERINC5 overlap those required for CD4 and tetherin downmodulation, in accordance with the AP-2 dependence of these functions (35, 45, 49, 50), SERINC5 antagonism was separable from other Nef functions. By engineering an infectious molecular clone of SIV that is selectively susceptible to SERINC5, we show that SERINC5 antagonism is required for optimal virus replication in cells expressing this restriction factor, including primary rhesus macaque lymphocytes.

Nef is a pleiotropic lentiviral accessory protein that plays an important role in immune evasion and pathogenesis through the downregulation of a number of cellular proteins from the surface of virus-infected cells (2, 3, 34, 35, 38, 51–54). As an adaptor for clathrin-mediated endocytosis, Nef stabilizes interactions of the cytoplasmic domains of these cargo proteins with subunits of the AP-1 or AP-2 complexes (45, 48, 49, 55). Whereas MHC I downmodulation by Nef is dependent on the formation of a

FIG 9 Phenotypic characterization of *SIV_{mac239nefAV}* in primary cells. The infectivities of viruses produced in primary $CD4^+$ T cells (A and F) and the downmodulation of CD4 (B and G), CD3 (C and H), MHC I (D and I), and tetherin (E and J) from the surface of infected $CD4^+$ T cells were compared. (A) The supernatant was collected from ConA-activated, CD8-depleted lymphocytes after 6 days of infection with *SIV_{mac239}*, *SIV_{mac239Δnef}*, and *SIV_{mac239nefAV}*, and the amount of virus present was determined by a p27 antigen capture ELISA. TZM-bl cells were infected with equivalent amounts of each virus (0.5 ng p27), and luciferase induction was measured 48 h later. RLU, relative luciferase units. (B to E) CD4, CD3, MHC I, and tetherin (BST-2) staining on the surface of virus-infected ($CD4^{lo}$ Gag^+) cells was measured by flow cytometry. Data are shown for one representative experiment (A to E) and for the average values from four independent experiments with $CD4^+$ T cells from each animal (F to J). (F) Average data are normalized to the infectivity of wild-type *SIV*. (G and H) Surface staining on infected cells was compared to that on uninfected cells ($CD4^{hi}$ Gag^-) in the same sample, and the average was normalized to *SIV_{mac239Δnef}*. Error bars indicate standard deviations of the means for the six animals, and *P* values were determined using two-tailed unpaired *t* tests with Welch's correction (ns, not significant).

trimolecular complex with the MHC class I cytoplasmic tail and the $\mu 1$ subunit of AP-1 (56, 57), CD3, CD4, tetherin, and SERINC downmodulation occurs through the formation of similar complexes with AP-2 (45, 48, 49, 55). In the case of tetherin, *SIV* Nef induces the refolding of a helix in the $\beta 2$ subunit of AP-2 to form an interface with the

FIG 10 Comparison of virus replication in CD4⁺ T cell lines and primary lymphocytes. CEMx174 cells (A), SupT1-CCR5 cells (B), and primary CD4⁺ T lymphocytes (C to E) were infected with equivalent amounts of SIV_{mac}239, SIV_{mac}239Δ*nef*, and SIV_{mac}239*Nef*_{AV}. Virus replication was monitored by the accumulation of SIV p27 in the cell culture supernatant by an antigen capture ELISA.

cytoplasmic domain of simian tetherin (48). This interface extensively overlaps sequences that contribute to interactions with SERINC5 (48). Nevertheless, we were able to separate SERINC5 and tetherin antagonism by a pair of amino acid changes (N72A and P73V) in the N-terminal domain of Nef. The introduction of these substitutions into SIV resulted in a nearly complete loss of the ability to counteract SERINC5 but did not affect tetherin antagonism or the downmodulation of tetherin from the surface of virus-infected cells. CD3 and MHC I downmodulation was also unaffected. However, although SIV_{mac}239*Nef*_{AV} retained the ability to downmodulate CD4, a small but significant increase in CD4 staining was observed relative to cells infected with wild-type SIV. Thus, while the N72A and P73V substitutions selectively disrupt SERINC5 antagonism without affecting at least three other functions of Nef, these results are consistent with a partial impairment of CD4 downmodulation.

A few substitutions that impair anti-SERINC5 activity were also found to disrupt tetherin antagonism and CD4 downmodulation, but not MHC I downmodulation. These include substitutions at positions in the N-terminal and flexible loop domains. Substitutions in the flexible loop have previously been reported to interfere with binding to AP-2 and to accordingly disrupt AP-2-dependent functions (45, 49). Although AP-2 interactions with the N-terminal domain of SIV Nef have not been reported,

mutations within the N-terminal region of HIV-1 SF2 Nef were recently shown to impair CD4 downmodulation and SERINC5 antagonism (58). Thus, in addition to the flexible loop region of Nef, which has been demonstrated to physically interact with subunits of the AP-2 complex, similar interactions with the N-terminal domain may also contribute to AP-2-dependent functions (45, 49, 58).

Sequence conservation is generally a good indicator of selective pressure to maintain a specific protein structure or function. Although the asparagine residue at position 72 of Nef is present in only approximately 35% of SIV genomes, the proline residue at position 73 is highly conserved in over 94% of SIV genomes (59). The conservation of P73 is consistent with an important role for this residue in counteracting SERINC5 and with the contribution of SERINC5 antagonism to viral fitness. However, while the N72A and P73V substitutions disrupted SERINC5 antagonism without the loss of the other AP-1- and AP-2-dependent functions assayed in this study, it is worth acknowledging that these residues may participate in Nef functions that were not tested or are yet to be identified.

Phylogenetically diverse retroviruses have evolved mechanisms to counteract SERINC5, suggesting that this factor represents a significant barrier to virus replication (2, 3, 22, 27, 28, 60). However, unlike other restriction factors such as APOBEC3G, TRIM5 α , and tetherin, which have acquired numerous species-specific differences and polymorphisms as a consequence of ongoing evolutionary conflict with viral pathogens (17, 19, 20, 24, 25, 61, 62), SERINC5 is well conserved and does not appear to be evolving under similar selective pressure (22). This has led some to question the physiological relevance of SERINC5 as a barrier to virus replication. To address the impact of SERINC5 on lentiviral replication, we performed a mutational analysis of the SIV_{mac}239 Nef protein to identify substitutions in Nef that disrupt SERINC5 antagonism without affecting other well-established Nef functions. This information was used to engineer an infectious molecular clone of SIV with a pair of amino acid changes in the N-terminal domain of Nef that uncouple SERINC5 antagonism from other functional activities of this accessory protein. These substitutions were sufficient to impair spreading infection in a CD4⁺ T cell line and in primary CD4⁺ T cells that express SERINC5, in accordance with an important role for Nef in counteracting this factor to promote efficient lentiviral replication.

MATERIALS AND METHODS

Plasmids. Full-length proviral DNA clones for wild-type SIV_{mac}239 and SIV_{mac}239 Δ nef were described previously (63, 64). Mutations in Nef were introduced into the bicistronic expression vector pCGCG-SIV_{mac}239 Nef by standard procedures for PCR-based site-directed mutagenesis as described previously (45). To construct proviral DNA clones with mutations in Nef, the required mutagenesis was first performed in the SIV_{mac}239 hemiviral constructs p239SpE3' and p239SpSp5'. The 5' and 3' hemiviruses were then digested with SphI and XhoI and ligated together to create the full-length proviral clone. To clone human and rhesus SERINC5, total RNA was extracted from the respective peripheral blood mononuclear cells (PBMCs), and SERINC5 cDNA was synthesized and cloned between NotI and BamHI sites in the pQCXIH vector. Sequences encoding the hemagglutinin (HA) epitope tag were introduced into the fourth extracellular loop of SERINC5 between amino acids 290 and 291 (SERINC5-iHA) as described previously (13). Sequences of all the plasmids were confirmed by Sanger sequencing.

Cell culture. Jurkat T antigen (JTAG) cells and JTAG-derived knockout cell lines were provided by Heinrich Gottlinger, University of Massachusetts Medical School, Worcester, MA (3). The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 270 nM amphotericin B, and 2 mM L-glutamine (R10). 293T cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 270 nM amphotericin B, and 2 mM L-glutamine (D10). TZM-bl cells were obtained from the NIH AIDS reagent program and cultivated in D10 (65, 66). C8166-SEAP cells (67) were maintained in R10 supplemented with 50 μ g/ml G418. CEMx174 cells (68) and SupT1-CCR5 cells (provided by James Hoxie, University of Pennsylvania, Philadelphia, PA) were maintained in R10. Rhesus macaque PBMCs were isolated from whole blood on a Ficoll-Hypaque gradient and cultured in R10. Concanavalin A (ConA)-activated PBMCs were maintained in R10 supplemented with 20 U/ml interleukin 2 (IL-2). To create stable cell lines expressing rhesus or human SERINC5 with an internal HA epitope tag, 293T cells were transduced with retroviral particles carrying the respective pQCXIH vector and selected and maintained in D10 supplemented with 100 μ g/ml hygromycin B.

SERINC5 antagonism. Assays to measure the antagonism of SERINC5 have been described previously (32, 69). Briefly, JTAG cells and JTAG SERINC3 knockout (S3KO) cell lines were cotransfected with

the SIV_{mac}239Δ*nef* proviral DNA clone (450 ng) along with pCGCG constructs that express wild-type Nef or Nef mutants (50 ng). All transfections were performed in 48-well plates with 5×10^4 cells using GenJet Jurkat transfection reagent (Signagen Laboratories) according to the manufacturer's instructions. At 3 days posttransfection, the virus released into the supernatant was collected by pelleting the cells, and the amount of virus present in the supernatant was quantified by an SIV p27 antigen capture ELISA. The equivalent of 0.5 ng of p27 was used to infect TZM-bl reporter cells (1×10^4) or C8166-SEAP cells (1×10^4) in triplicate wells of 96-well plates. At 3 days postinfection, luciferase activity from TZM-bl cells or secreted-alkaline phosphatase activity from the supernatant of C8166-SEAP cells was quantified as a measure of infectivity. After subtracting reporter activity in the absence of Nef, the anti-SERINC5 activity of the Nef mutants was calculated as a percentage of reporter activity relative to that in the presence of wild-type Nef. Although 1 μM amphotericin B was recently shown to alleviate the sensitivity of HIV-1 pseudovirions to SERINC5 (5), the lower concentration of this antifungal agent in our cell culture medium (270 nM) did not have a detectable effect on the sensitivity of *nef*-deleted SIV to human or macaque SERINC5. To measure the downmodulation of SERINC5 by Nef, 293T cells stably expressing rhesus or human SERINC5 with an internal HA epitope tag were transfected with 100 ng of SIV_{mac}239 or *nef*-deleted SIV_{mac}239 or SIV_{mac}239_{Δ*nef*} proviral DNA clones. At 2 days posttransfection, the cells were dispersed with phosphate-buffered saline (PBS)–10 mM EDTA, stained with an Alexa Fluor 647-conjugated anti-HA monoclonal antibody (16B12 clone; BioLegend), and fixed in 2% paraformaldehyde. The cells were then permeabilized and stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to the SIV capsid protein (55-2F12 clone). After gating on SIV-expressing (Gag⁺) cells, the mean fluorescence intensity of HA staining was determined.

CD4, MHC class I, tetherin, and CD3 downmodulation. CD4 and MHC class I downmodulation was assessed in TZM-bl and 293T cells, respectively. Cells plated in 24-well plates were transfected with 1 μg of bicistronic pCGCG constructs that express wild-type Nef, or Nef mutants, and green fluorescent protein (GFP) from an internal ribosome entry site (IRES). Twenty-four hours later, 293T cells were stained with an allophycocyanin (APC)-conjugated monoclonal antibody to MHC I (W6/32 clone; BioLegend), and TZM-bl cells were stained with a peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibody to CD4 (OKT4 clone; BioLegend). After gating on GFP⁺ cells, the mean fluorescence intensity (MFI) of CD4 and MHC I expression was determined. CD3, CD4, BST-2, and MHC I downmodulation was also assessed in rhesus macaque primary CD4⁺ lymphocytes infected with SIV_{mac}239, SIV_{mac}239Δ*nef*, or SIV_{mac}239_{Δ*nef*}. Cells were stained with antitetherin APC (clone RS38E), anti-CD3 phycoerythrin (PE)-cyanine-based fluorescent 594 (CF594) (clone Sp34-2), anti-CD4 PE-Cy7 (OKT4), anti-MHC I PE, and near-infrared (IR) live/dead cell stain (Invitrogen) and fixed in 2% paraformaldehyde. The cells were then permeabilized and stained with an FITC-conjugated monoclonal antibody to the SIV capsid protein p27 (55-2F12 clone). The MFIs of the staining for the respective proteins were determined by gating on SIV-infected (Gag⁺) cells. Data were collected using an LSR II flow cytometer (BD) and analyzed using FlowJo software version 9.7.7 (TreeStar).

Virus release assay. 293T cells (5×10^4) were plated in 24-well plates and transfected the next day with *nef*-deleted proviral DNA (100 ng), and a tetherin expression construct, pcDNA3-rBST-2.2 (50 ng), together with 100 ng of Nef expression constructs or an empty vector (pCGCG) were provided in *trans*. Control transfections to assess virus release in the absence of tetherin were performed for each experiment. Differences in the amounts of plasmid DNA in each transfection were offset by the addition of the empty vector. At 48 h posttransfection, the amount of virus released into the supernatant was measured by an SIV p27 antigen capture ELISA, and virus release was expressed as a percentage of the maximal particle release in the absence of tetherin.

Immunoblotting. 293T cell lysates were prepared by harvesting in 1× SDS sample buffer. Virions released into the cell culture supernatant were recovered by centrifugation at $20,000 \times g$ for 2 h at 4°C and resuspended in 1× SDS sample buffer. Samples were heated for 5 min at 95°C, separated by electrophoresis on 12% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T) for 1 h and probed with the following primary antibodies. Tetherin was detected using the monoclonal antibody clone E-4 (Santa Cruz Biotechnology) at a dilution of 1:1,000. SIV Gag proteins p27 and p55 were detected with the monoclonal antibody 183-H12-5C (70, 71) (NIH AIDS reagent program) at a dilution of 1:500. SIV Nef was detected using polyclonal SIV antisera from an SIV_{mac}239-infected rhesus macaque at a dilution of 1:100. KK41 monoclonal antibody at a dilution of 1:1,000 was used to detect the gp41 subunit of SIV Env. SERINC5-iHA was detected using HA.11 mouse monoclonal antibody (Sigma) at a dilution of 1:1,000. Hsp90 was detected using the F-8 monoclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:1,000. After rinsing the membranes three times in PBS-T, the blots were probed with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Li-Cor) at a dilution of 1:3,000 or an HRP-conjugated anti-human secondary antibody (Thermo Scientific) at a dilution of 1:1,000. The blots were then rinsed three more times with PBS-T, treated with Clarity Western ECL substrate (Bio-Rad), and imaged using a GE ImageQuant LAS4000 image reader.

Virus replication assays. Rhesus macaque PBMCs were activated with ConA (5 μg/ml). Three days later, the cells were washed and cultured in R10 supplemented with 20 U/ml IL-2. Two days later, the activated PBMCs were depleted of CD8⁺ cells using anti-CD8 immunomagnetic beads. Activated lymphocytes (2×10^6 cells) were infected with wild-type SIV_{mac}239, SIV_{mac}239Δ*nef*, and SIV_{mac}239_{Δ*nef*}. The day after inoculation, the cells were washed to remove the inoculum and cultured in R10 supplemented with 20 U/ml IL-2. Virus replication was monitored by measuring the accumulation of SIV p27 in the cell culture supernatant by an antigen capture ELISA.

Statistical analyses. CD4 and MHC I downregulation in cells transfected with Nef mutants was compared to that in cells transfected with wild-type Nef. The relative infectivities of SIV in the presence of Nef mutants in JTAG cells with knockout mutations in SERINC3 were compared to the infectivities of viruses produced in the presence of wild-type Nef. Statistical analyses of tetherin antagonism by the Nef mutants were performed by comparing virus release relative to wild-type SIV Nef using Student's *t* test. The expressions of surface molecules by virus-infected lymphocytes were compared using an unpaired *t* test with Welch's correction.

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