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VIRUS-CELL INTERACTIONS

A Conserved Acidic-Cluster Motif in SERINC5 Confers Partial Resistance to Antagonism by HIV-1 Nef

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ABSTRACT The cellular protein SERINC5 inhibits the infectivity of diverse retroviruses, and its activity is counteracted by the glycosylated Gag (glycoGag) protein of murine leukemia virus (MLV), the S2 protein of equine infectious anemia virus (EIAV), and the Nef protein of human immunodeficiency virus type 1 (HIV-1). Determining the regions within SERINC5 that provide restrictive activity or Nef sensitivity should inform mechanistic models of the SERINC5/HIV-1 relationship. Here, we report that deletion of the conserved sequence EDTEE, which is located within a cytoplasmic loop of SERINC5 and which is reminiscent of an acidic-cluster membrane trafficking signal, increases the sensitivity of SERINC5 to antagonism by Nef, while it has no effect on the intrinsic activity of the protein as an inhibitor of infectivity. These effects correlated with enhanced removal of the ΔEDTEE mutant relative to that of wild-type SERINC5 from the cell surface and with enhanced exclusion of the mutant protein from virions by Nef. Mutational analysis indicated that the acidic residues, but not the threonine, within the EDTEE motif are important for the relative resistance to Nef. Deletion of the EDTEE sequence did not increase the sensitivity of SERINC5 to antagonism by the glycoGag protein of MLV, suggesting that its virologic role is Nef specific. These results are consistent with the reported mapping of the cytoplasmic loop that contains the EDTEE sequence as a general determinant of Nef responsiveness, but they further indicate that sequences inhibitory to as well as supportive of Nef activity reside in this region. We speculate that the EDTEE motif might have evolved to mediate resistance against retroviruses that use Nef-like proteins to antagonize SERINC5.

IMPORTANCE Cellular membrane proteins in the SERINC family, especially SERINC5, inhibit the infectivity of retroviral virions. This inhibition is counteracted by retroviral proteins, specifically, HIV-1 Nef, MLV glycoGag, and EIAV S2. One consequence of such a host-pathogen "arms race" is a compensatory change in the host antiviral protein as it evolves to escape the effects of viral antagonists. This is often reflected in a genetic signature, positive selection, which is conspicuously missing in *SERINC5*. Here we show that despite this lack of genetic evidence, a sequence in SERINC5 nonetheless provides relative resistance to antagonism by HIV-1 Nef.

KEYWORDS HIV-1, Nef, SERINC

Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus, encoding accessory genes that evolved to enhance viral fitness in response to host selective pressures (1). The accessory gene *nef* accelerates *in vivo* pathogenesis and the progres-

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sion to AIDS, despite being nonessential for viral propagation in cell culture (2–4). Expression of the Nef protein occurs early during the viral replication cycle, preceding the expression of structural proteins, such as the envelope glycoprotein (Env), and preceding virion assembly (5). Posttranslational myristoylation on an N-terminal glycine residue enables Nef to associate with lipid membranes (6), where it modulates the trafficking of host proteins to promote immune evasion. Nef activities include downregulation of the HIV receptor CD4 (6) and major histocompatibility complex class I (MHC-I) from the cell surface (7). To modulate CD4, Nef uses a dileucine-based motif to recruit components of the cellular protein sorting machinery, specifically, the clathrin adaptor protein complex 2 (AP-2), to induce the endocytosis of CD4 and, ultimately, to target it to multivesicular bodies (MVBs) and lysosomes for degradation (8–11). CD4 modulation is also an activity of the HIV accessory protein Vpu; together, the activities of Vpu and Nef prevent CD4 and Env from interacting in the virion producer cell. This ensures the proper maturation of Env, preventing CD4 from inhibiting virion infectivity and from triggering the exposure of CD4-dependent epitopes in Env that are good targets for host humoral immunity (12–17). In contrast to the above-described consensus regarding CD4, two mechanisms have been proposed for the Nef-mediated modulation of MHC-I: (i) Nef utilizes the clathrin adaptor AP-1 to bind newly synthesized and antigen-loaded MHC-I molecules within the *trans*-Golgi network (TGN) to target them for lysosomal degradation (18), and (ii) Nef accelerates the internalization of MHC-I from the cell surface via a phosphatidylinositol 3-kinase-regulated and ARF6-mediated pathway to promote the sequestration of MHC-I within the TGN (19). Either of these mechanisms would cause a reduction of MHC-I molecules at the cell surface and the resistance of HIV-1-infected cells to killing by cytotoxic T lymphocytes (20).

Another highly conserved activity of Nef is the enhancement of virion infectivity (21, 22). This activity is preserved among *nef* alleles obtained from HIV-1-infected individuals at different stages of disease progression, suggesting that it is important for both transmission and persistent infection (23). The infectivity effect is dependent on specific regions within Nef, all of which are also required for the modulation of CD4, including the above-noted dileucine-based motif. Components of the cellular endocytic machinery (AP-2, Dynamin 2, and clathrin) are also required (24, 25). Nef must be expressed within virion producer cells to enhance infectivity; its presence in target cells and in virions is dispensable (26, 27). These observations led to the hypothesis that Nef prevents a cell surface infectivity-inhibiting factor from incorporating into virions.

Serine incorporator 3 (SERINC3) and SERINC5 were identified to be such factors; they are transmembrane proteins that incorporate into virions and that inhibit the infectivity of retroviruses (28, 29). Nef counteracts this by removing SERINC3 and SERINC5 from the plasma membrane in an AP-2-dependent manner (29). Nef's ability to enhance infectivity depends quantitatively on the relative sensitivity or resistance of the tested Env protein to inhibition by the SERINCs: the Nef effect is the greatest when the matching Env protein is sensitive to SERINC5 (28, 29). This sensitivity, in turn, appears to correlate directly with the openness of the Env trimer and, consequently, with the sensitivity of the Env to neutralizing antibodies that are selectively active against more open trimers (30).

SERINC3 and SERINC5 are members of a conserved family of proteins whose cellular function includes phospholipid biosynthesis, specifically, the incorporation of serine into membrane lipids (31). Nonetheless, SERINC5 does not appear to alter the lipid composition of virions (32). Instead, SERINC5 seems to inhibit the fusion of virions with target cells, potentially by functionally inactivating sensitive Env trimers (33). Nef prevents the incorporation of SERINC5 into virions, presumably by physically interacting with SERINC5, stimulating its endocytosis, and sending it toward lysosomal degradation (34). SERINC5 antagonists have been identified in retroviruses other than HIV and simian immunodeficiency virus (SIV). These include the glycosylated Gag (glycoGag) protein of murine leukemia virus (MLV) (35) and the S2 protein of equine infectious anemia virus (EIAV) (36). While Nef and glycoGag are structurally unrelated, the mechanisms by which they counteract SERINC5 are similar: endocytosis and

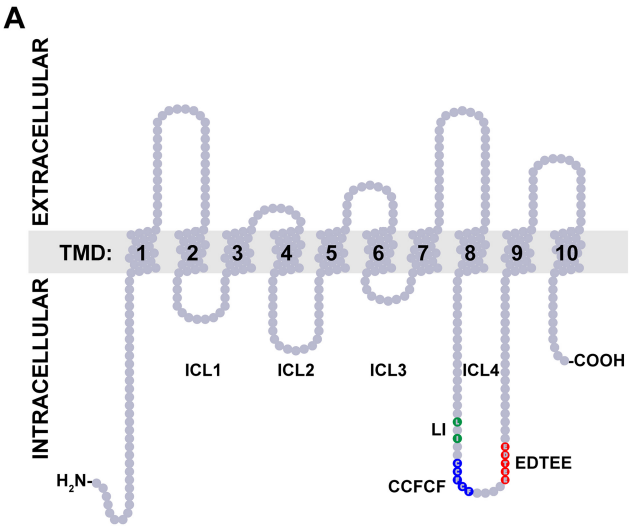
lysosomal degradation (37). Despite this scenario of host-pathogen conflict between the SERINCs and retroviral proteins, *SERINC3* and *SERINC5* do not appear to be under positive selection at the gene level, at least not to the extent observed for other antiretroviral restriction factors, such as *TRIM5*α or *BST-2* (38).

The goal of this study was to determine whether a potential membrane trafficking signal in SERINC5, reminiscent of an acidic-cluster sorting motif, supported the activity of Nef. This sequence, EDTEE, is within the same cytoplasmic loop that has recently been shown to determine Nef sensitivity (39). The hypothesis that this sequence would support Nef activity is consistent with the roles of sequences reminiscent of sorting motifs in other Nef targets, such as the key tyrosine in the cytoplasmic domain of the class I MHC α chain and the dileucine motif in the cytoplasmic domain of CD4 (8, 40, 41). Paradoxically, we found that rather than supporting Nef activity, the EDTEE sequence instead provided a degree of protection against Nef: a lack of the EDTEE sequence enhanced Nef activity as an antagonist of SERINC5. The relatively increased infectivity of virions produced in the presence of Nef and SERINC5 lacking the EDTEE sequence correlated with the more efficient exclusion of SERINC5 from virions and the more efficient downregulation of cell surface SERINC5 by Nef. This enhanced response phenotype appeared to be specific to Nef proteins; deletion of the EDTEE sequence slightly impaired rather than enhanced the activity of glycoGag as an antagonist of SERINC5. We speculate that the EDTEE region could have evolved to render Nef-like proteins less active as SERINC5 antagonists.

RESULTS

The EDTEE sequence reduces the sensitivity of SERINC5 to HIV-1 Nef. An alignment between various SERINC5 proteins reveals an acidic sequence (EDTEE; Fig. 1) that is highly conserved among mammals and that is located in a long, predicted cytoplasmic loop (designated intracytoplasmic loop 4 [ICL4]). The EDTEE sequence is reminiscent of an acidic-cluster membrane trafficking signal (54), so we hypothesized that it might be a Nef-response sequence and support Nef activity. To test this, we cotransfected HEK293 cells or Jurkat TAg (JTAg) cells that lack endogenous SERINC3 and -5 (*SERINC3/5*-knockout [KO] cells [*S3/5*-KO cells]) with pNL4-3 (an HIV-1 infectious molecular clone) or its Nef-negative counterpart (pNL4-3ΔNef) along with increasing amounts of plasmids expressing either hemagglutinin (HA)-tagged SERINC5 (pBJ5-SERINC5-iHA) or SERINC5-HA from which EDTEE was deleted (SERINC5-HA ΔEDTEE; pBJ5-SERINC5-iHA ΔEDTEE). The virions produced were partially purified by centrifugation through a 20% sucrose cushion, and their infectivity was measured using an infectious center (IC) assay. The IC assay values were divided by the concentration of the p24 capsid antigen, and the IC assay value/p24 concentration ratios were normalized to those for the no-added-SERINC5 control, setting that control value to 100% for both the wild-type (WT) and the Nef-negative viruses. The latter normalization removed from the presented data differences between the infectivity of wild-type and Nef-negative viruses that were not due to the experimental expression of SERINC5 or related mutants by transfection. For our HEK293 cells, the *nef* infectivity phenotype in the absence of plasmid-mediated expression of SERINC5 was 6- to 10-fold and was presumably due to the endogenous expression of SERINC family members (data not shown). For the Jurkat TAg *SERINC3/5*-KO cells, the *nef* infectivity phenotype in the absence of plasmid-mediated expression of SERINC5 was approximately 2-fold, despite the genetic disruption of these two *SERINC* family members (Fig. 2E).

As expected, we observed a dose-dependent antiviral effect of SERINC5, which was greater when virions were produced in the absence of Nef (Fig. 2A). Surprisingly, deletion of the EDTEE sequence enhanced the sensitivity of SERINC5 to Nef, but it did not affect the inhibitory activity of the protein in the absence of Nef (Fig. 2A). This enhanced Nef response was observed using either HEK293 or Jurkat TAg *SERINC3/5*-KO cells as virion producers. Although the difference in sensitivity to Nef was not clearly attributable to differences in SERINC5 protein expression (Fig. 2B, which shows representative results of an experiment in which Jurkat cells were used to produce virions), a subtle influence of the deletion on protein expression was apparent in the dose-



B

ICL4

	332 350 355 364 389
Homo sapiens (Human) NP001167543.1	STTRSSSDALQG-RYAAPELEIARCCFCFS-PGGEDTEEQQPGKEGPRVIYDEKKGTVYI
Pan troglodytes (Chimpanzee) XP016808565.1	.....-.....-.....
Pan paniscus (Bonobo) XP003822322.1	.....-.....-.....
Pongo abelii (Sumatran Orangutan) XP024103145.1	.....-.....-.....
Gorilla gorilla gorilla (West Lowland Gorilla) XP018869110.1	.....-.....-.....
Macaca mulatta (Rhesus Macaque) XP014995821.1	.....-.....-.....Q.....
Cercocebus atys (Sooty Mangabe) XP011944576.1	.....-.....-.....Q.....
Chlorocebus sabaeus (African Green Monkey) XP007974831.1	.....-.....-.....Q.....
Saimiri boliviensis (Black-capped Squirrel Monkey) XP003920853.1	.....-.....-.....K.....V
Callithrix jacchus (Common Marmoset) XP017825310.1	.....-.....-.....K.....
Mus musculus (House Mouse) NP766176.1	.....S-..G.....V.....G-.D.....NV.K.....S
Xenopus tropicalis (Western Clawed Frog) XP002940241.1	.....R.-..TP..T.M.....C.RSD.D.E..RTEKRG.QE.G...E.S.I.C
Danio rerio (Zebrafish) NP998679.1	...KR..A...VY.NDM..N.R.....WV-DDT..YDDEKT-SG.QN.K...RD....S
Equus caballus (Horse) XP023473861.1	....A.....-.....V.....G-.D.....NV.....S

FIG 1 An acidic-cluster motif (EDTEE) is highly conserved within mammalian SERINC5. (A) Predicted topology of SERINC5 showing 10 transmembrane domains and 6 cytoplasmic domains, 4 of which form loops. The EDTEE sequence is shown in red and is found within the long intracytoplasmic loop 4 (ICL4). Hydrophobic residues (LI; green) and a cysteine-rich putative palmitoylation site (blue) are also indicated. (B) Amino acid sequence alignment of SERINC5 ICL4. The conserved EDTEE acidic-cluster motif is shown in red.

response Western blot assay data: the ΔEDTEE mutant seemed to be slightly underexpressed, particularly in the absence of Nef. Consistent with the infectivity data, deletion of the EDTEE sequence caused enhanced exclusion of SERINC5 from virions by Nef (Fig. 2B and C, which show representative results of an experiment in which Jurkat cells were used to produce virions and quantitative data from three experiments). We confirmed that a 55-kDa form of SERINC5, while the minority species in cells, is the predominant form in virions, an effect due to the selective incorporation into virions of a form of the protein modified by complex glycans (58). The quantitative data suggested that the ΔEDTEE mutant was slightly less efficiently incorporated into virions than wild-type SERINC5 in the absence of Nef; the data also indicated that the ΔEDTEE mutant was excluded from virions by Nef about 2-fold more efficiently than wild-type SERINC5 (Fig. 2C and D). These data support the current model that the Nef-mediated exclusion of SERINC5 from virions correlates with enhanced infectivity (28, 29). The data also indicate that the EDTEE sequence within SERINC5 provides a degree of resistance to Nef activity; Nef is more active in the absence of this sequence.

To determine whether a window for the EDTEE effect exists in the case of endogenous SERINC, we revisited the phenotype of *nef* (in NL4-3) both in Jurkat TAg cells, which express large amounts of *SERINC5* mRNA, and in Jurkat TAg *SERINC3/5*-knockout cells (generated by gene editing). Consistent with previous reports, we observed a 32-fold Nef effect when virions were produced from JTAg cells but only a 2.5-fold effect when virions were produced from the JTAg *SERINC3/5*-KO cells (Fig. 2D). Importantly, the activity of Nef as a SERINC antagonist was incomplete: the knockout of *SERINC3* and

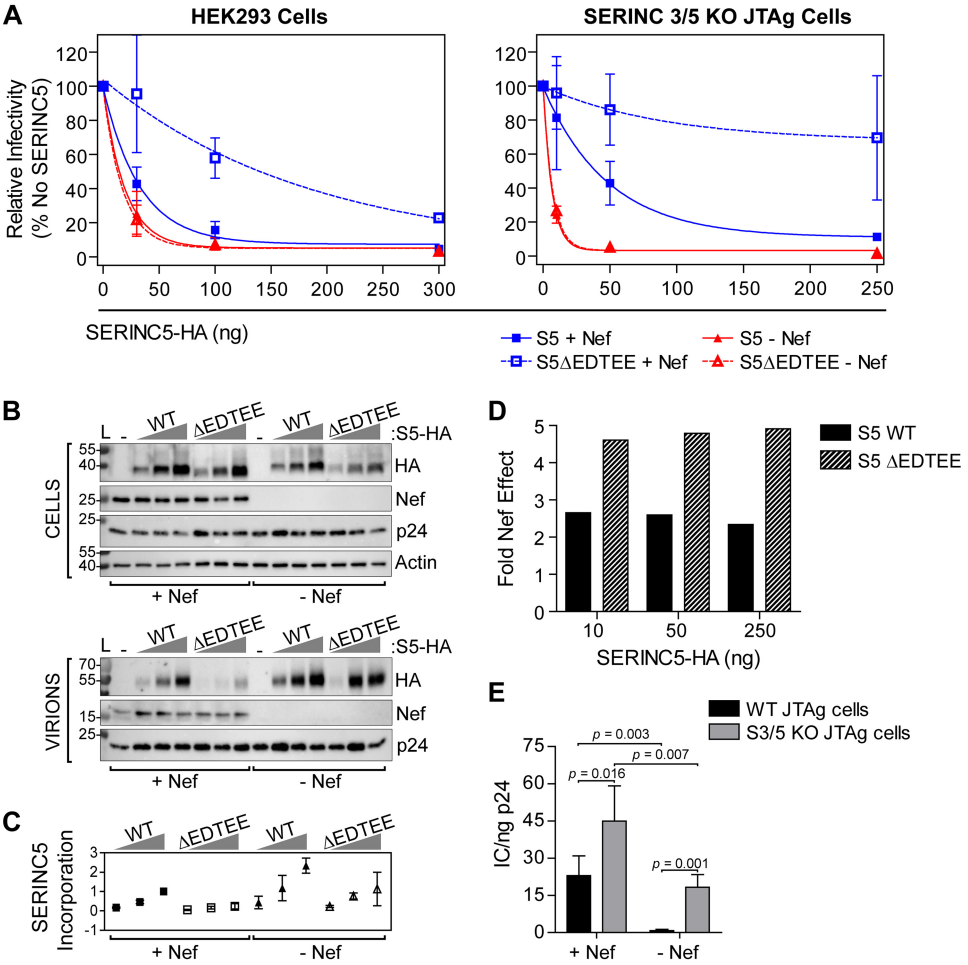


FIG 2 The EDTEE sequence within SERINC5 is not necessary for antiviral activity but confers relative resistance to Nef. (A) HEK293 cells or Jurkat TAg cells lacking SERINC3 and SERINC5 (JTAg *S3/5*-KO) were transfected to express NL4-3 (WT) or a *nef*-negative mutant (NL4-3ΔNef) and increasing amounts of SERINC5-HA or a mutant lacking the acidic-cluster motif (SERINC5-iHA ΔEDTEE), as indicated. The produced virions were partially purified by centrifugation through a sucrose cushion and used to infect HeLa P4.R5 cells, which express an LTR–*β*-galactosidase indicator. Forty-eight hours later, the cells were stained with X-Gal, and the infectious centers (IC) were imaged and quantified. The numbers of ICs per milliliter were divided by the concentration (in nanograms per milliliter) of p24 antigen measured in the virion preparations by ELISA. The numbers of infectious centers per nanogram were normalized to the values for the no-added-SERINC5 control for each viral genotype (+Nef, wild type; −Nef, Nef negative). Data are presented as the mean percent relative infectivity; error bars are the standard deviations (SD) from 2 (HEK293 cells) and 3 (JTAg *S3/5*-KO cells) experiments. (B) Protein from whole JTAg *S3/5*-KO cell lysates and virions from the experiment whose results are presented in panel A were subjected to SDS-PAGE and Western blotting. The membranes were probed with antibodies to detect SERINC5 (HA), Nef, p24, and *β*-actin. Virions produced by JTAg *S3/5*-KO cells and analyzed in the experiment whose results are presented in panel A were normalized by their p24 content before SDS-PAGE. The membranes were probed for p24/p55, Nef, and SERINC5 (HA). Nef is cleaved within virions by the viral protease, yielding a 20-kDa C-terminal product (57). L, molecular mass ladder. The numbers to the left of the gel are molecular masses (in kilodaltons). (C) The intensities of the bands for SERINC5 were quantified and normalized to the intensity of the bands for p24. Data are presented as the fold SERINC5 incorporation relative to that for the condition with 250 ng SERINC5 plus Nef (set equal to 1) and are from 3 experiments. (D) Data are presented as the fold virion exclusion activity of Nef at each amount of SERINC5 plasmid transfected. (E) Infectivity of wild-type Nef (+Nef) or *nef*-negative (−Nef) virions produced from wild-type JTAg cells or *SERINC3/5*-knockout cells, expressed as the ratio of the numbers of IC per milliliter/p24 antigen concentration (in nanograms per milliliter).

SERINC5 increased the infectivity of virions produced from JTAg cells by 2-fold in the presence of Nef (Fig. 2E). This incomplete antagonism creates the potential for the EDTEE effect to be operative in the case of endogenous protein.

SERINC5 ICL4 is phosphorylated by casein kinase II *in vitro* and binds the *μ* subunits of AP-1 and AP-2. We reported the presence of a phosphoserine acidic cluster (PSAC) motif of the sequence SGASDEED in the cytoplasmic loop (loop 10) of

A

SERINC5 ICL4:

$_{332}$SSTTRSSSDALQGRYAAPELEIARCCFCFSPGGEDTEEQQPGKEGPRVIYDEKKGTVYI$_{389}$

LCMS Phosphopeptide	Location	Mascot score
EDTEEQQPGKEGPRVIYD + Phospho (ST)	366	35.5
STTRSSSD + Phospho (ST)	333	29.9
STTRSSSD + Phospho (ST)	334	25.0
STTRSSSD + Phospho (ST)	337	22.5

B

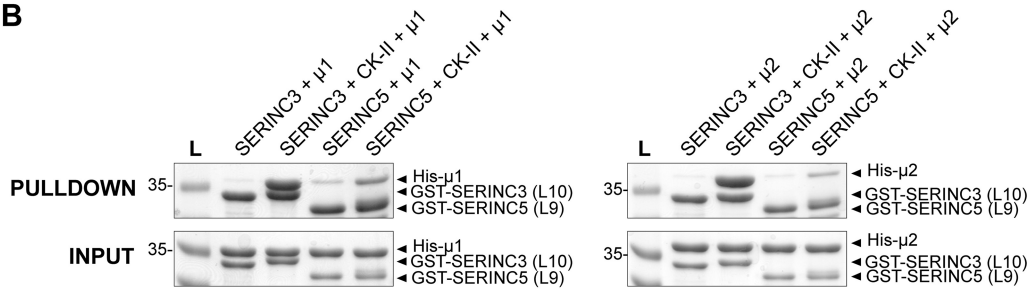


FIG 3 Phosphorylated SERINC5 ICL4 binds to the medium (μ) subunit of AP-1 and AP-2. (A) Sequence of SERINC5 ICL4. GST-SERINC5 ICL4 was coexpressed with casein kinase II (CK-II) in *E. coli*, purified, and analyzed by liquid chromatography-mass spectrometry (LC-MS). Phospho-peptide sequences are shown; the sites of serine-threonine (ST) phosphorylation are highlighted in red, and the residue locations are indicated. Mascot scores for the peptide matches are shown. (B) Phospho-SERINC5 ICL4 (here labeled L9, for loop 9) binds weakly to $\mu1_{CTD}$ and $\mu2_{CTD}$ relative to phospho-SERINC3 loop 10 (L10). GST-SERINC3 loop 10 (a positive control) and GST-SERINC5 ICL4 (L9) fusion proteins were expressed in *E. coli* either with or without CK-II and tested for binding to $\mu1_{CTD}$ and $\mu2_{CTD}$ in GST-pulldown assays. SDS-PAGE gels were stained with Coomassie blue. The numbers to the left of the gels are molecular masses (in kilodaltons).

SERINC3 analogous to the loop that contains the EDTEE sequence in SERINC5. Unlike the EDTEE sequence, the SGASDEED sequence has no impact on sensitivity to Nef, despite the fact that the serines of this sequence are under positive selection (38, 54). The SGASDEED sequence of SERINC3 has potential as a membrane sorting or trafficking sequence, because it binds the medium (μ) subunits of clathrin adaptors AP-1 ($\mu1$) and AP-2 ($\mu2$) in a serine phosphorylation-dependent manner (54). Here we observed that when the recombinant SERINC5 loop containing the EDTEE sequence (ICL4) was coexpressed as a glutathione *S*-transferase (GST) fusion protein together with casein kinase II in *Escherichia coli*, the threonine of the EDTEE sequence, as well as upstream serines in the loop, were phosphorylated (Fig. 3A). Moreover, phosphorylated SERINC5 ICL4 bound to recombinant $\mu1$ and $\mu2$ *in vitro* (Fig. 3B), although the extent of binding was less than that in the case of SERINC3 loop 10. These data indicate that the EDTEE sequence has the potential for threonine phosphorylation. The data also support a potential role for this sequence as a μ-binding clathrin adaptor sorting signal.

The acidic residues within the EDTEE motif but not the threonine affect Nef sensitivity. We next asked whether the acidic nature of the SERINC5 EDTEE motif is the determinant of sensitivity to Nef. We created SERINC5 mutants that either lacked acidic residues (EDTEE mutated to AATAA [SERINC5-AATAA]) or preserved acidic residues (EDTEE mutated to EDAEE [SERINC5-EDAEE]) and tested their restrictive activity and sensitivity to Nef. SERINC5-AATAA, but not SERINC5-EDAEE, was characterized by an enhanced sensitivity to Nef, which was similar to the phenotype of SERINC5-ΔEDTEE in both HEK293 and JTAg *SERINC3/5*-KO cells (Fig. 4A). All the SERINC5 mutants were as restrictive as the wild-type protein; that is, they inhibited the infectivity of virions produced in the absence of Nef as effectively as wild-type SERINC5, despite the fact that the expression of the ΔEDTEE and AATAA mutants (but not the EDAEE mutant) seemed slightly reduced. Overall, these data suggest that the relative acidity or negative charge of the SERINC5 EDTEE region affects sensitivity to Nef but not intrinsic restrictive activity. The data also indicate that the threonine alone is not a substantial determinant

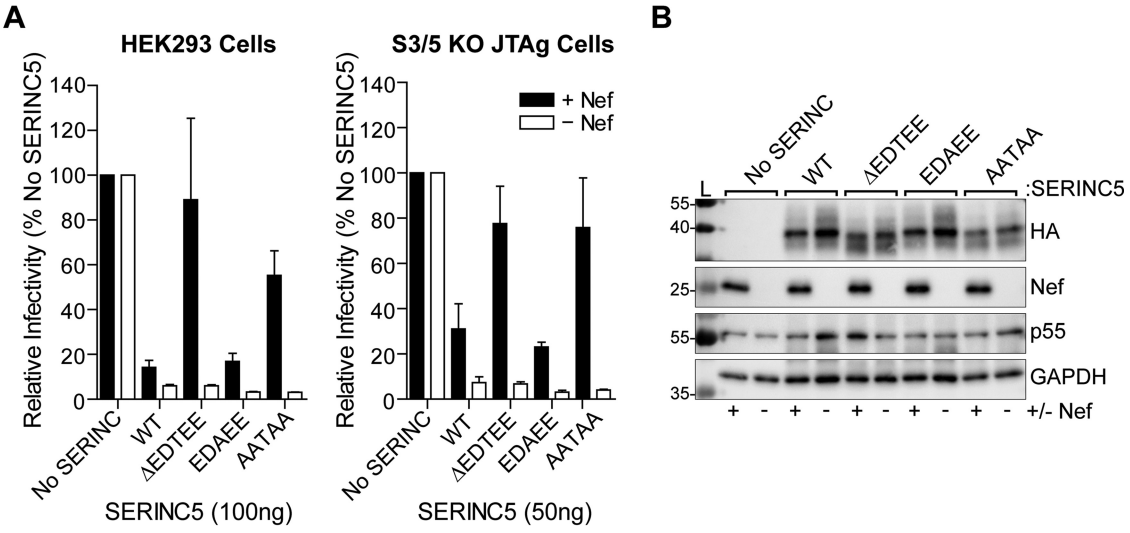


FIG 4 The acidic residues within the SERINC5 EDTEE sequence, but not the threonine residues, are important for resistance to Nef. (A) HEK293 cells or JTAg *S3/5*-KO cells were transfected with pNL4-3 or pNL4-3ΔNef and the indicated amounts of plasmid expressing either WT SERINC5 or the following mutants: the ΔEDTEE, EDAEE, or AATAA mutant. The virions were harvested and infectivity assays were performed in HeLa P4.R5 cells, as described in the legend to Fig. 2. The numbers of infectious centers per nanogram were normalized to those for the no-added-SERINC5 control for each viral genotype. Data are presented as the mean percent relative infectivity; error bars are the SD from 2 (HEK293 cells) and 3 (JTAg *S3/5*-KO cells) experiments. (B) Protein derived from whole JTAg *S3/5*-KO cell lysates from the experiment whose results are presented in panel A were subjected to SDS-PAGE and Western blotting. The membranes were probed with antibodies to detect SERINC5 (HA), Nef, p24/p55, and GAPDH. The numbers to the left of the gel are molecular masses (in kilodaltons).

of sensitivity to Nef, even though it would contribute to the negative charge of the region if it were phosphorylated.

The enhanced sensitivity of EDTEE-deleted SERINC5 to Nef requires residues in ICL4 previously associated with Nef responsiveness. We tested the hypothesis that the increased sensitivity of the EDTEE deletion mutant to Nef requires nearby residues in ICL4 that were reportedly required for Nef responsiveness (39, 59). We evaluated the LI/AA (residues 350 and 352) and CCC/SSS (residues 355, 356, and 358) substitutions in the loop; the LI residues were mapped genetically and could be determinants of the interaction with Nef (39), whereas the CCC residues are potential sites of palmitoylation (59). These substitutions were made in the context of either an intact or a deleted EDTEE sequence (Fig. 5). When the EDTEE sequence was intact, the LI/AA and CCC/SSS substitutions reduced Nef responsiveness, albeit incompletely. When the EDTEE sequence was deleted, the reduction in Nef responsiveness caused by the LI/AA and CCC/SSS substitutions was greater, likely due at least partly to the larger Nef effect observed in the ΔEDTEE mutant context. These data indicate that the enhanced response of EDTEE-deleted SERINC5 to Nef involves the same hydrophobic residues and potential palmitoylation sites in ICL4 identified in previous studies. The data confirm that sequences inhibitory to Nef responsiveness (EDTEE) as well as supportive of Nef responsiveness (LI and CCC) reside in this region.

Deletion of the EDTEE sequence enhances Nef's ability to downregulate SERINC5. Nef co-opts endocytic machinery, namely, AP-2 and clathrin, to downregulate SERINC5 from the plasma membrane (28, 29). Based on our virologic data, we hypothesized that the downregulation of SERINC5 by Nef would be increased by deletion of the EDTEE sequence. To test this, we cotransfected cells with pNL4-3 or pNL4-3ΔNef together with the SERINC5-iHA expression plasmids and measured surface SERINC5 levels by immunofluorescent staining and flow cytometry. The mutant SERINC5 constructs (ΔEDTEE, AATAA, and EDAEE) were similarly expressed at the cell surface in Jurkat TAg *SERINC3/5*-KO cells in the absence of Nef; their expression levels were slightly less than those of wild-type SERINC5 (e.g., about 15% less in the case of the ΔEDTEE construct) (Fig. 6A, top). SERINC5-ΔEDTEE and SERINC5-AATAA, but not

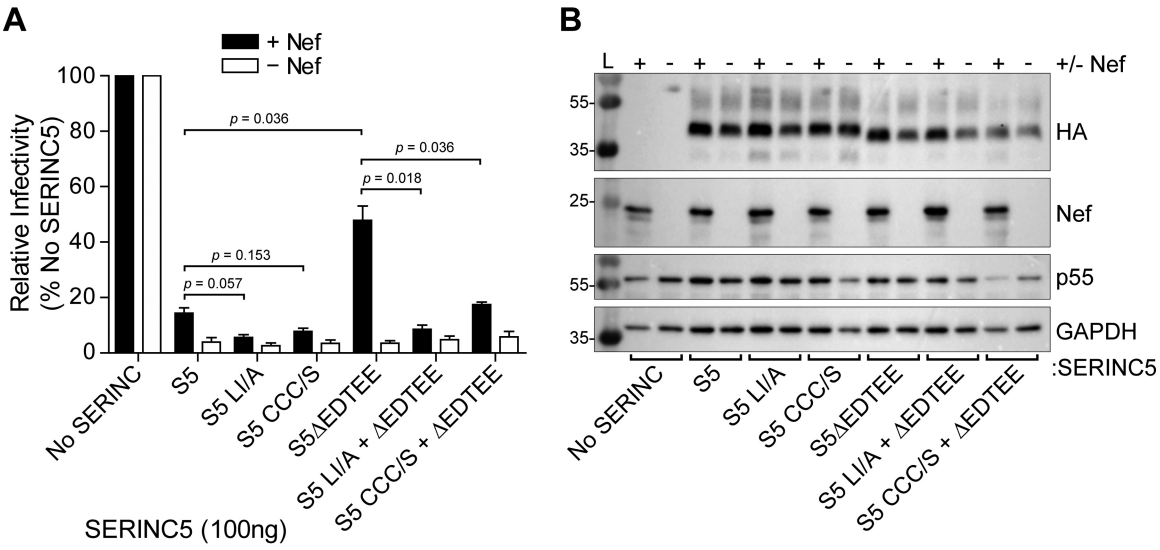


FIG 5 Enhanced Nef responsiveness of EDTEE-deleted SERINC5 requires hydrophobic and cysteine residues in ICL4. (A) HEK293 cells were transfected with pNL4-3 (+Nef) or pNL4-3ΔNef (−Nef) and 100 ng of plasmid expressing WT SERINC5 or the following mutants: the LI/AA, CCC/SSS, ΔEDTEE, ΔEDTEE plus LI/A, or ΔEDTEE plus CCC/SSS mutant. Virion infectivity was measured as described in the legend to Fig. 2. The data are representative of those from three independent experiments. (B) SDS-PAGE Western blot of samples from the experiment whose results are presented in panel A. The antibodies used were to HA (SERINC5), Nef, p24/p55, and GAPDH. The numbers to the left of the gel are molecular masses (in kilodaltons).

SERINC5-EDAEE, were downregulated more efficiently than wild-type SERINC5 in both JTAg *SERINC3/5* and HEK293 cells (Fig. 6). These results are consistent with the virologic data and support the correlation between the downregulation of cell surface SERINC5 and the enhancement of infectivity by Nef. The data further support the suggestion that the EDTEE sequence provides relative resistance to the Nef-mediated modulation of SERINC5.

Deletion of the EDTEE sequence does not enhance the Nef/SERINC5 interaction, as measured using BiFC. We used a previously reported bimolecular fluorescence complementation (BiFC) assay (34, 46) to test the hypothesis that the interaction of Nef and SERINC5 is enhanced in the absence of the EDTEE sequence. In this assay, the two proteins of interest were fused to either the N or C terminus of Venus (yellow fluorescent protein). A fluorescent signal is generated if the two proteins interact, enabling the quantitative measurement of protein-protein interactions within living cells. Here, we fused the N terminus of Venus (VN) to the C terminus of either wild-type NL4-3 Nef or a myristoylation signal mutant incapable of associating with membranes (Nef-G2A) (6). We also fused the C terminus of Venus (VC) to the C terminus of either SERINC5, SERINC5-ΔEDTEE, SERINC5-EDAEE, or SERINC5-AATAA. These constructs were used to transfect HEK293 cells either singly or in pairs, and the relative fluorescence was measured by flow cytometry 24 h later. We detected a 4-fold relative increase in fluorescence when Nef-VN and SERINC5-VC were coexpressed compared to the fluorescence when either protein alone was expressed (Fig. 7A). This increase in the fluorescent signal was lost when SERINC5-VC was paired with Nef-G2A-VN, consistent with the notion that Nef requires a membrane association to interact with SERINC5. We did not detect an increased interaction signal when Nef-VN was paired with either SERINC5-ΔEDTEE-VC or SERINC5-AATAA-VC relative to the signal obtained with SERINC5-VC (Fig. 7A). No differences in the expression of these fusion proteins was detected by Western blotting (Fig. 7B). These data indicate that deletion of the SERINC5 EDTEE sequence does not enhance its interaction with Nef, as measured by this assay.

SERINC5 ICL4 stimulates the interaction of Nef with AP-2 *in vitro*, but the interaction is not markedly enhanced by deletion of the EDTEE sequence. We next sought to determine whether deletion of the EDTEE sequence enhances the formation of a ternary complex including Nef, ICL4 of SERINC5, and the clathrin adaptor AP-2. We

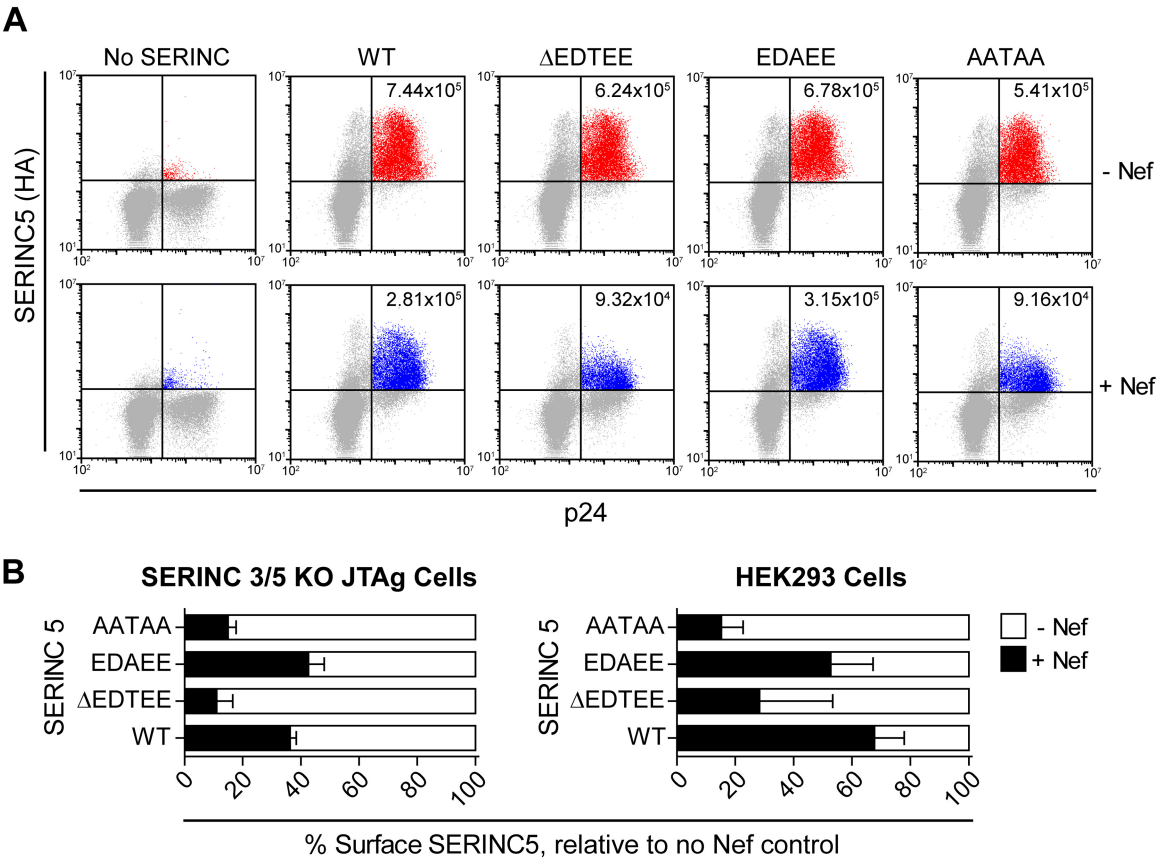


FIG 6 Mutation of the EDTEE sequence enhances the downregulation of SERINC5 from the cell surface by Nef. JTAg *S3/5*-KO or HEK293 cells were transfected with pNL4-3 (+Nef) or pNL4-3ΔNef (−Nef) and either 250 ng (JTAg *S3/5*-KO) or 100 ng (HEK293) of plasmid expressing WT SERINC5 or EDTEE mutant ΔEDTEE, EDAEE, or AATAA. The cells were stained for surface SERINC5 (HA, Alexa Fluor 647) and intracellular p24 (FITC). (A) Representative two-color dot plots showing the surface expression of SERINC5 (HA) in p24-positive JTAg *S3/5*-KO cells (with or without Nef). The numbers in the plots are the mean fluorescence intensity (MFI) of the entire p24-positive population. (B) The mean fluorescence intensity of surface SERINC5 (HA) in p24-positive cells was quantified with or without Nef. Data are presented as the mean percent mean fluorescence intensity normalized to that for the no-Nef control; error bars are the SD from 2 (JTAg *S3/5*-KO cells) or 4 (HEK293 cells) experiments.

produced recombinant proteins containing NL4-3 Nef (residues 25 to 206) fused via a long flexible linker to either SERINC5 ICL4 (residues 332 to 387) or a SERINC5 ICL4 ΔEDTEE mutant (Fig. 8A). Nef without ICL4 was used as a control. Each of these proteins was fused to maltose-binding protein (MBP) to enhance their solubility. The binding of these proteins to a recombinant, $\mu 2$ C-terminal domain ($\mu 2_{CTD}$)-truncated AP-2 heterotetramer *in vitro* was analyzed by pulldown assays using GST-tagged AP-2 mixed with either MBP-Nef, MBP-Nef-SERINC5 ICL4 or MBP-Nef-SERINC5 ICL4 ΔEDTEE (Fig. 8B). ICL4 strikingly stimulated the pulldown of Nef with the $\mu 2_{CTD}$-truncated AP-2 complex, a result consistent with the notion that this cytoplasmic loop is a Nef response sequence and that Nef and ICL4 together bind efficiently to AP-2. However, we detected little or no influence of the EDTEE sequence in this assay: the Nef-ICL4 fusion protein did not clearly bind more efficiently to AP-2 when the EDTEE sequence was deleted (Fig. 8B). These results suggest that the EDTEE sequence does not interfere with the formation of a complex of Nef, SERINC5 ICL4, and $\mu 2_{CTD}$-truncated AP-2 when assessed using recombinant proteins *in vitro*.

Role of the EDTEE sequence in the antagonism of SERINC5 by glycoGag. Based on our results with Nef, we hypothesized that the EDTEE sequence might affect the antagonism of SERINC5 by the glycosylated Gag (glycoGag) protein of Moloney murine leukemia virus (M-MLV). MLV glycoGag counteracts SERINC3 and SERINC5 and rescues the infectivity of *nef*-deficient HIV-1 (35). The majority of the extracellular domain of

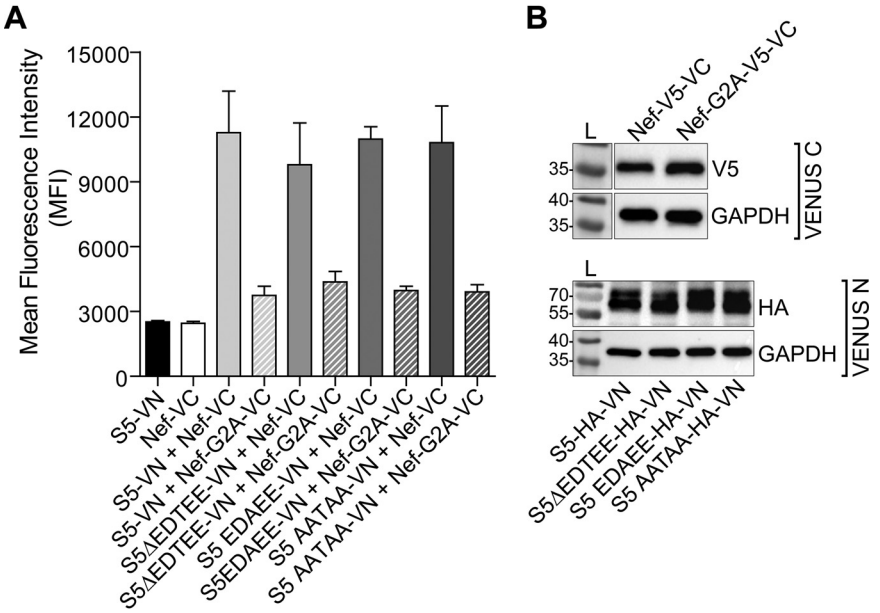


FIG 7 The intracellular interaction between Nef and SERINC5, measured by bimolecular fluorescence complementation (BiFC), is not affected by the EDTEE sequence. (A) HEK293 cells were transfected with plasmid constructs expressing Nef-Venus C terminus (VC), Nef-G2A-VC, SERINC5-Venus N terminus (VN), SERINC5-ΔEDTEE-VN, SERINC5-EDAEE-VN, or SERINC5-AATAA-VN either singly or in the pairs indicated in the figure. Twenty-four hours later, the fluorescence intensity (FL1) was measured by flow cytometry. The data are presented as the mean fluorescence intensity of the Venus signal; error bars are the SD from 3 independent experiments. (B) Protein from whole-cell lysates from a representative experiment was subjected to SDS-PAGE and Western blotting. The membranes were probed with anti-V5 (Nef-VC), HA (SERINC5-VN), or GAPDH (loading control) antibodies. The numbers to the left of the gel are molecular masses (in kilodaltons).

M-MLV glycoGag is dispensable for this activity (60). We therefore used a minimal, active truncated form of glycoGag that contains the N-terminal 189 residues (gg189) to test the ability of glycoGag to rescue the infectivity of HIV-1 lacking Nef in the presence of either SERINC5 or the SERINC5 mutant lacking the EDTEE sequence. For these

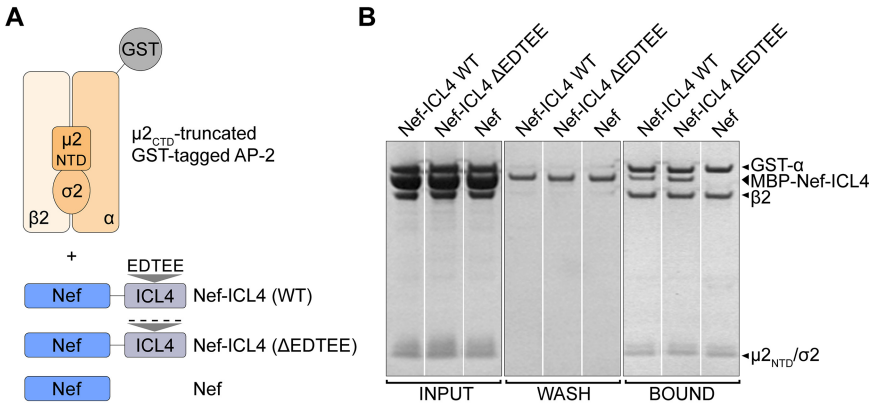


FIG 8 SERINC5 (ICL4) stimulates the interaction of Nef with AP-2 *in vitro* independently of the EDTEE sequence. (A) Schematic of the recombinant protein constructs used to study the formation of a Nef-SERINC5 ICL4 complex *in vitro*. A heterotetrameric AP-2 complex core was expressed with a GST tag on the α subunit; the C-terminal two-thirds of the μ2 subunit was deleted, as were the appendage domains of the α and β2 subunits. Nef was fused to SERINC5 ICL4 (with or without the EDTEE sequence) via a linker peptide; these proteins were further fused to the maltose-binding protein (MBP) as a solubility tag (not shown). MBP-Nef alone was used as a control. (B) GST-pulldown assay assessing the binding of Nef-SERINC ICL4 (with or without the EDTEE sequence) or Nef alone to the truncated AP-2 core *in vitro*. The input protein mixtures, the protein(s) washed through the GST matrix, and the protein(s) that remained bound to the GST-matrix were run on an SDS-PAGE gel and stained with Coomassie blue. NTD, N-terminal domain.

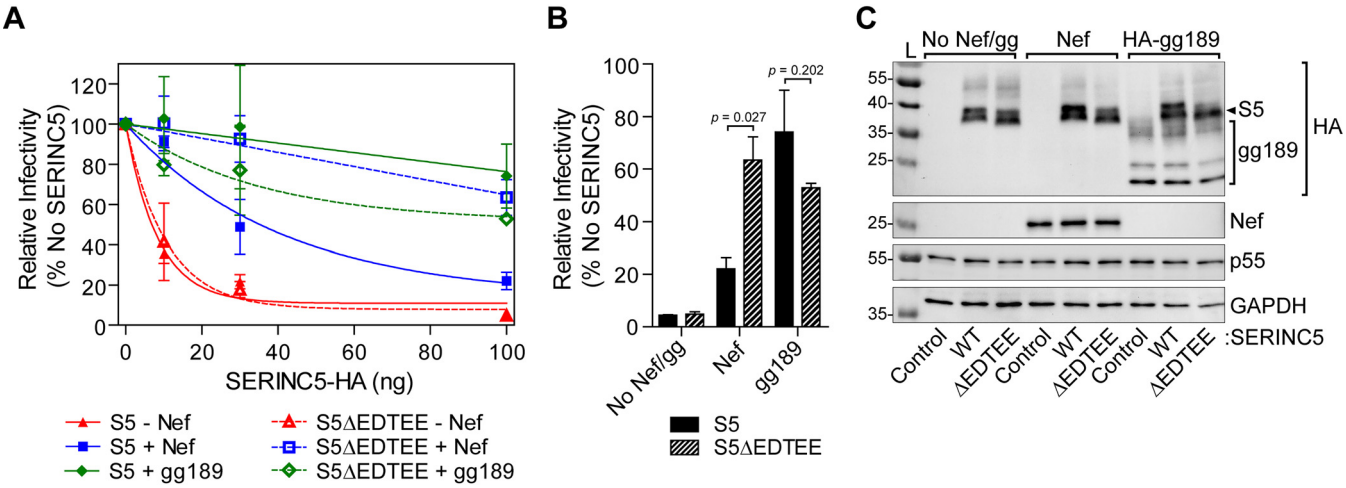


FIG 9 Deletion of the EDTEE sequence does not enhance the activity of MLV glycoGag as an antagonist of SERINC5. (A) HEK293 cells were cotransfected with plasmid constructs encoding the indicated amounts of SERINC5-iHA and either *env*- and *nef*-deficient (−Nef) or *env*-deficient (+Nef) HIV-1 provirus. Env expression was provided in *trans* by an NL4-3-derived construct (pVRE). An HA-tagged minimally active glycoGag (HA-gg189) was also provided in *trans*. Virions were harvested at 24 h posttransfection, partially purified by centrifugation through a sucrose cushion, and used to infect HeLa-TZM-bl luciferase indicator cells. Luciferase activity was measured at 48 h postinfection and normalized to that of the p24 antigen (relative light units [RLU]/concentration of p24 [in nanograms per milliliter] ratio). Data are expressed as percent infectivity relative to that for the no-added-SERINC control for each condition (with or without Nef or glycoGag). Error bars indicate the standard deviations from 2 independent experiments. (B) Comparison of relative infectivity of virions in the absence or the presence of 100 ng SERINC5-iHA (*P* values derived from Student's *t* test are indicated). (C) Protein from whole-cell lysates was subject to SDS-PAGE and Western blotting. The membranes were probed with antibodies to detect SERINC5 (HA), glycoGag (gg189, HA), Nef, p55 (Gag), and GAPDH. The numbers to the left of the gel are molecular masses (in kilodaltons). No Nef/gg, expression of neither Nef nor gg189.

experiments, HIV-1 Env was provided in *trans* in the virion producer cells, in order to abrogate syncytium formation in the target cells, which was strikingly exaggerated when the absence of Nef was complemented by glycoGag and Env was encoded in the viral genome (data not shown). HeLa-TZM-bl indicator cells were used for luminometric measurement of infectivity, as this provided a more sensitive method for measuring the infectivity of the pseudovirions. We confirmed that, as shown above, the EDTEE sequence provided relative resistance to Nef when infectivity was measured using this modified experimental design (Fig. 9). As reported previously, glycoGag efficiently antagonized the activity of SERINC5 as an inhibitor of infectivity (Fig. 9). Unlike Nef, however, the activity of glycoGag was not enhanced when the EDTEE sequence of SERINC5 was deleted; in contrast, its activity against the EDTEE mutant was slightly diminished. These data suggest that the role of the EDTEE sequence in SERINC5 is Nef specific, despite the fact that the cellular cofactors involved in SERINC antagonism by Nef and glycoGag are similar (28, 29, 60).

Deletion of the EDTEE sequence enhances the response of SERINC5 to diverse Nef proteins. To exclude the possibility that the enhanced Nef responsiveness of the EDTEE-deleted SERINC5 was peculiar to NL4-3 Nef, we evaluated three other Nef proteins: Nef from clone SF2 (47, 48) and Nefs from two inferred transmitted-founder (TF) viruses (49–53). We used an experimental format similar to that with which we evaluated glycoGag as described above: the Env and Nef proteins were provided in *trans* to an Δ*env* Δ*nef* genome at the time of virion production, and the infectivity of the pseudovirions was measured using HeLa-TZM-bl cells (Fig. 10). All the tested Nef proteins were more active against the EDTEE mutant than against wild-type SERINC5. These data indicate that the EDTEE sequence reduces the sensitivity of SERINC5 to diverse HIV-1 Nef proteins.

Deletion of the EDTEE sequence does not affect the subcellular localization of SERINC5 in either the presence or the absence of Nef. We used immunofluorescence microscopy to determine whether the EDTEE sequence affected the subcellular localization of SERINC5. We imaged wild-type SERINC5 or the ΔEDTEE mutant in both HEK293 and HeLa P4.R5 cells (Fig. 11). We tested the effects of the Nef protein of strain

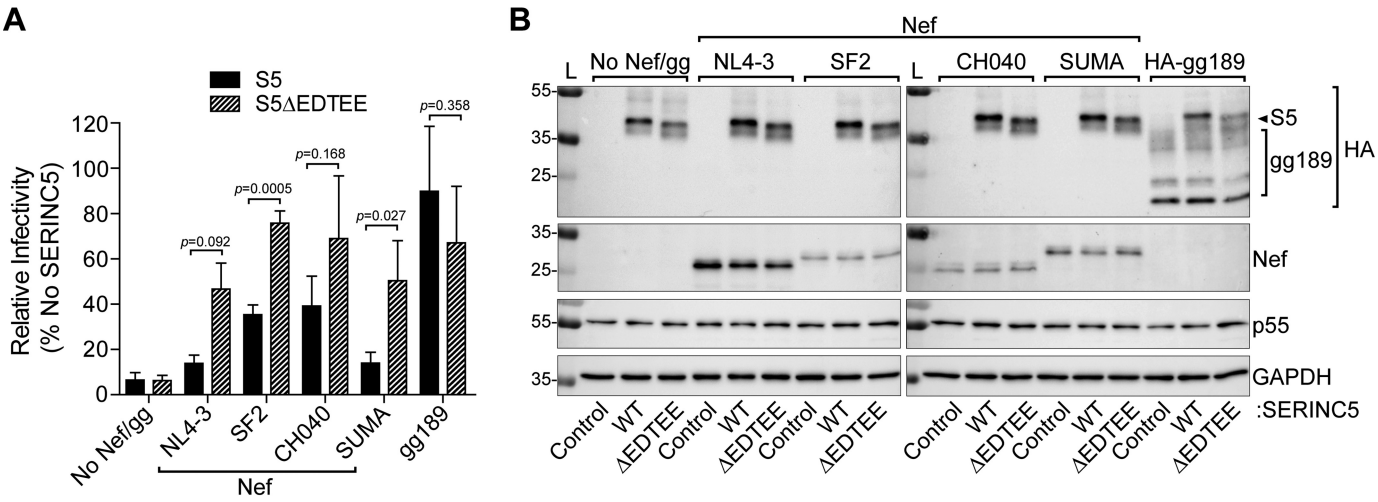


FIG 10 Deletion of the EDTEE sequence enhances the activity of diverse Nef proteins. (A) HEK293 cells were cotransfected with plasmid constructs encoding SERINC5-iHA (100 ng) and *env*- and *nef*-deficient HIV-1 provirus. Env expression was provided in *trans* by an NL4-3-derived construct (pVRE). FLAG-tagged Nef derived from NL4-3, SF2, or TF clones CH040 and SUMA and an HA-tagged minimally active glycoGag (HA-gg189) were also provided in *trans* (via 100 ng plasmid). Virions were harvested at 24 h posttransfection, partially purified by centrifugation through a sucrose cushion, and used to infect HeLa-TZM-bl luciferase indicator cells. Luciferase activity was measured at 48 h postinfection and normalized to that of the p24 antigen (relative light units [RLU]/concentration of p24 [in nanograms per milliliter] ratio). Data are expressed as percent infectivity relative to that for the no-added-SERINC control (100%) for each condition (with or without Nef or glycoGag). Error bars indicate the standard deviations from 3 independent experiments. *P* values derived from Student's *t* test are indicated. (B) Protein from whole-cell lysates was subject to SDS-PAGE and Western blotting. The membranes were probed with antibodies to detect SERINC5 (HA), glycoGag (gg189, HA), Nef, p55 (Gag), and GAPDH. The numbers to the left of the gel are molecular masses (in kilodaltons).

NL4-3 (Nef_{NL4-3}) or strain SF2 (Nef_{SF2}) using an experimental design similar to that of the infectivity assays for which the results are shown in Fig. 10. In the absence of Nef, wild-type SERINC5 and the ΔEDTEE mutant were indistinguishable; both displayed a pattern of nuclear envelope and cytoplasmic staining reminiscent of that for the endoplasmic reticulum; occasional cells also showed staining at the cell perimeter, consistent with staining of the plasma membrane. Neither Nef_{NL4-3} nor Nef_{SF2} markedly changed this distribution, precluding a determination of whether the EDTEE sequence rendered SERINC5 less effectively relocalized by Nef within the cell.

DISCUSSION

We initially hypothesized that the acidic cluster within the long cytoplasmic loop of SERINC5—the sequence EDTEE in ICL4—might function as a protein-sorting motif in concert with HIV-1 Nef and thus support Nef activity as a SERINC5 antagonist. Instead, our data indicate that Nef is more effective as a SERINC5 antagonist in the absence of the EDTEE sequence. A SERINC5 mutant lacking this sequence or a mutant in which the acidic residues were replaced with alanines was more effectively antagonized by Nef at the levels of counteraction of SERINC5-mediated inhibition of infectivity, downregulation of SERINC5 from the cell surface, and exclusion of SERINC5 from virions. These data are consistent with the model of surface downregulation and virion exclusion of SERINC5 as the basis for the Nef-mediated enhancement of infectivity, although this mechanism of counteraction by Nef has recently been challenged by studies of endogenous SERINC5 (61). Our data also support the notion that the region of SERINC5 containing the EDTEE sequence—ICL4—determines Nef sensitivity.

In addition to the EDTEE sequence, ICL4 contains two hydrophobic residues, a leucine and an isoleucine, that were reported to be required for Nef responsiveness (39). More recently, putative palmitoylation sites in ICL4 have also been reported to be required (59). We confirmed that all of these residues contribute to Nef responsiveness, effects that were particularly evident when Nef activity was enhanced by deletion of the EDTEE sequence. These data are consistent with the conclusion that ICL4 determines Nef sensitivity and together indicate that the loop contains residues supportive of as well as inhibitory to Nef activity.

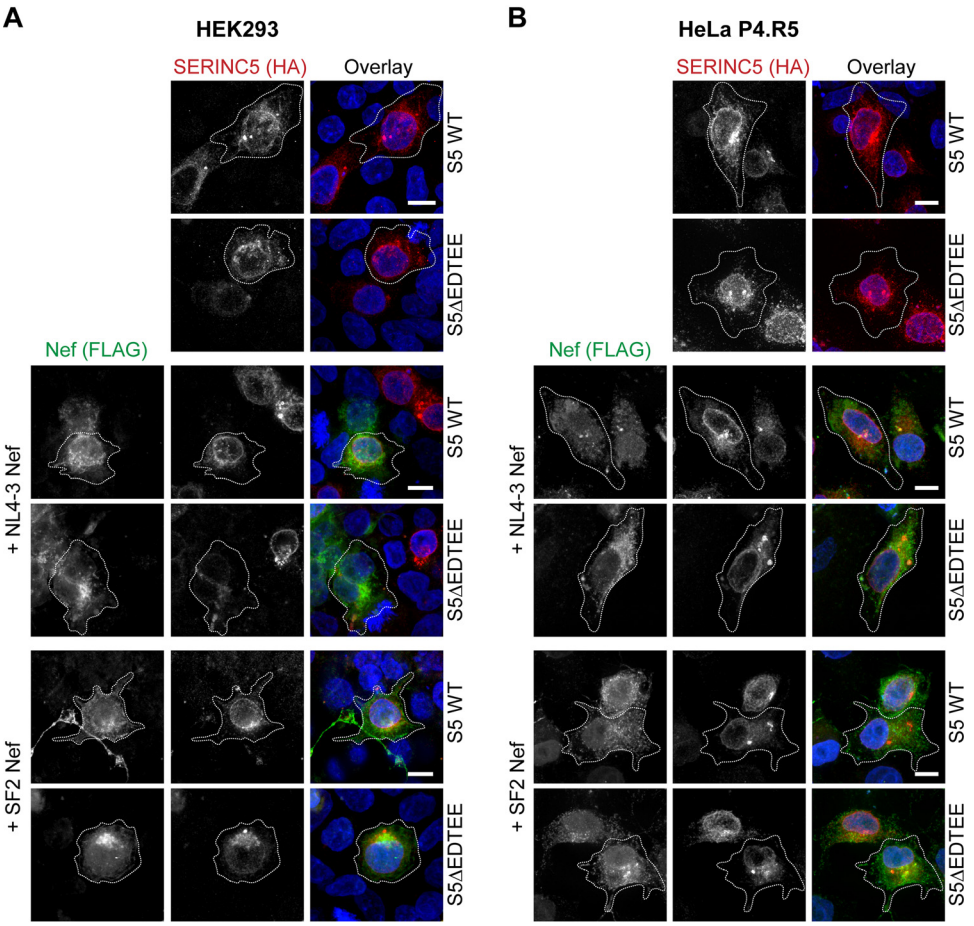


FIG 11 Subcellular localization of the EDTEE mutant in comparison to that of wild-type SERINC5. HEK293 (A) or HeLa P4.R5 (B) cells were cotransfected with plasmids encoding wild-type SERINC5-iHA or the ΔEDTEE mutant (100 ng) and *env*- and *nef*-deficient HIV-1 provirus. Env expression was provided in *trans* by an NL4-3-derived construct (pVRE). FLAG-tagged Nef derived from NL4-3 or SF2 was also provided in *trans*. The cells were fixed and stained for the HA epitope tag (SERINC5; shown in red) and for the FLAG epitope (Nef; shown in green). Nuclei were stained with DAPI. The images are projections of *z*-stacks obtained using wide-field fluorescence microscopy after deconvolution. The cell borders were outlined for clarity. Bars = 10 μm.

Why would a cytoplasmic loop of SERINC5 contain an inhibitory sequence like EDTEE? Although *SERINC5* does not seem to be under positive selection among primates, a genetic signature of host-pathogen conflict (38), we considered that the EDTEE sequence might have evolved to provide protection against diverse retroviruses and the SERINC antagonists that they encode. The retroviral accessory proteins Nef (found in HIVs and SIVs), glycoGag (found in MLV), and S2 (found in EIAV) are structurally unrelated proteins that all enhance viral infectivity by counteracting SERINC5 (28, 29, 35, 36). However, our data indicate that the EDTEE sequence is not inhibitory to the activity of glycoGag, suggesting that the impact of this sequence is Nef specific. This scenario weighs against the notion that the protein acquired the EDTEE sequence as a general defense against retroviral antagonists. It also implies that Nef has not yet optimally evolved to counteract SERINC5. Alternatively, or in addition, the importance of other Nef functions for viral fitness might preclude such evolution. An evolutionary constraint on Nef is supported by the observation that each of the four distinct Nef proteins evaluated here was a more effective SERINC5 antagonist when the EDTEE sequence was deleted.

How does the EDTEE sequence affect Nef responsiveness? One possible explanation is that the sequence is a membrane trafficking signal that directs SERINC5 away from Nef. Consistent with this possibility, we observed that phosphorylated ICL4 binds the μ

subunits of the clathrin adaptor complexes AP-1 and AP-2 *in vitro*, although this interaction seemed less robust than the interaction of the μ subunits with the analogous loop of SERINC3, loop 10, which contains a more typical phosphoserine acidic-cluster sequence (SGASDEED) (54). On the other hand, we found no evidence that deletion of the EDTEE sequence influences the subcellular localization of SERINC5. Another possibility is that the negative charge of the EDTEE sequence inhibits its interaction with Nef. This model is consistent with the requirement of the acidic residues for this phenotype; it might also be consistent with the presence of an acidic cluster in the N-terminal region of Nef, which might repel SERINC5. However, our measurement of the SERINC5/Nef interaction using bimolecular fluorescence complementation did not support this model: the interaction signal was unaffected by deletion of the EDTEE sequence. Moreover, a charge-repulsion model predicts that a Nef mutant in which the acidic cluster is neutralized would be more active as an antagonist of wild-type (EDTEE motif-containing) SERINC5, but we did not find that to be the case (data not shown).

Yet another possibility is that a subtle decrease in the steady-state and/or surface expression of the ΔEDTEE and AATAA mutants is sufficient to increase their apparent Nef responsiveness. While this possibility is conceivably correct, several lines of evidence weigh against it. First, the SERINC5 EDTEE mutants appeared to reach the plasma membrane, the presumed site of Nef counteraction via endocytosis, nearly as efficiently as the wild-type protein (as measured by flow cytometry). Second, none of our data suggest that the intrinsic restrictive activities of the mutant proteins were decreased (i.e., their activities against virus lacking *nef* were unaffected). Third, and perhaps most importantly, the ΔEDTEE mutant was not more responsive than wild-type SERINC5 to glycoGag; rather, the mutant appeared to be slightly less responsive. The observation that the effect of deleting the EDTEE sequence is opposite when testing the responsiveness to Nef versus that to glycoGag is inconsistent with the notion that the observed virologic phenotypes are consequences of the levels of protein expression.

The phenotype that we have investigated—that of an up-mutation for responsiveness to Nef—is, by definition, observable only if the antagonism of wild-type SERINC5 is incomplete. Incomplete antagonism can be caused by either an intrinsically low activity of the Nef protein or overexpression of SERINC5 to levels beyond what Nef can counteract. Our data comparing four different Nef proteins support the context-dependent variability of the ΔEDTEE phenotype. For example, SF2 Nef appears to be more active than NL4-3 Nef as a SERINC5 antagonist. While SF2 Nef is more active against the ΔEDTEE mutant than against wild-type SERINC5, the increase is not as great as that observed in the case of NL4-3 Nef (2.2-fold for SF2 Nef versus 3.7-fold for NL4-3 Nef), which is intrinsically less active than SF2 Nef as an antagonist of SERINC5. Accounting for overexpression is challenging; our data and those of others show that Nef is easily overcome by high levels of SERINC5 expression (62). Nonetheless, even with the smallest amount of exogenous SERINC5 tested in Jurkat T cells (see the results for the 10-ng plasmid amount in Fig. 2A, a condition in which NL4-3 Nef recovered infectivity to 80% of that for the no-SERINC5 condition), deletion of the EDTEE sequence still rendered SERINC5 more Nef responsive. These data suggest that the overexpression of SERINC5 to levels that overwhelm Nef is not necessary to see the EDTEE phenotype. Along a related line of reasoning, we observed that the antagonism of endogenous SERINC proteins by Nef is incomplete: genetic disruption of the *SERINC*3 and *SERINC*5 genes in Jurkat TAg cells increased the infectivity of virions expressed by *nef*-positive as well as *nef*-negative viral genomes. This incomplete Nef activity creates the potential for the EDTEE effect to be operative during the endogenous expression of SERINC5.

The question of exactly how the EDTEE motif in SERINC5 inhibits the activity of Nef as a SERINC5 antagonist remains open. Although our binding experiments using recombinant proteins *in vitro* do not clearly support the hypothesis that the EDTEE sequence inhibits the interaction between Nef-SERINC5 and the AP-2 clathrin adaptor complex, we have not yet attempted to assess this ternary interaction in the more

complex environment of human cells. Moreover, our constructs for the expression of heterotetrameric AP-2 *in vitro* lack the C-terminal domain of μ2 to which the EDTEE sequence might bind. A structural explanation of the presumed interaction between Nef, SERINC5 ICL4, and AP-2 might yet provide an answer for the currently enigmatic role of this motif. Alternatively, we are intrigued by the recent observation that ICL4 is palmitoylated and that ICL4 palmitoylation is required for optimal Nef responsiveness (59). Palmitoylation could enrich SERINC5 in membrane microdomains shared by Nef, or it could position ICL4 optimally to interact with Nef. Conceivably, the acidic EDTEE sequence could inhibit the palmitoylation-driven association of ICL4 with the plasma membrane by means of charge repulsion with membrane phospholipids, rendering the activity of Nef less efficient.

MATERIALS AND METHODS

Cells. HEK293 cells (obtained from Saswati Chatterjee) (42) and HeLa-TZM-bl cells (obtained from John Kappes via the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HeLa P4.R5 cells (obtained from Ned Landau) were maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 μg/ml puromycin. A leukemic T cell clone (Jurkat E6.1) lacking endogenous SERINC3 and SERINC5 (generated by CRISPR editing and termed JTAg *S3/5*-KO cells) and the parental JTAg cells were gifts from Heinrich Göttlinger (28). These cells were cultured in complete RPMI medium with 10% FBS and 1% penicillin-streptomycin.

Plasmids. The proviral plasmids pNL4-3 and pNL4-3ΔNef have been described previously (21, 43, 44). The pNL4-3-derived plasmids lacking *env* (DHIV) or lacking both the *env* and *nef* genes (DHIVΔNef) were gifts from Vicente Planelles (45). The plasmid pCINeo-VRE (pVRE) contains the sequence of NL4-3 from 9 bp upstream of the Rev start codon to the XhoI site in Nef (100 bp downstream of the Nef start codon) and harbors the sequences for Vpu, Rev, and Env. The empty vector pBJ5 and the pBJ5-HA-gg189 plasmid containing an HA-tagged minimal active truncated form (the N-terminal 189 residues) of MLV glycoGag were a gift from Massimo Pizzato. The plasmid pBJ5-SERINC5-iHA was a gift from Heinrich Göttlinger (28). This SERINC5 plasmid contains an HA tag located between residues 290 and 291 in extracellular loop 4 of the protein. pBJ5-SERINC5-iHA ΔEDTEE, pBJ5-SERINC5-iHA AATAA, pBJ5-SERINC5-iHA EDAEE, pBJ5-SERINC5-iHA L350A/I352A, pBJ5-SERINC5-iHA ΔEDTEE plus L350A/I352A, pBJ5-SERINC5-iHA C355S/C356S/C358S, and pBJ5-SERINC5-iHA ΔEDTEE plus C355S/C356S/C358S, containing mutations in the intracellular loop 4 (ICL4; residues 332 to 387) region of human SERINC5, were generated using site-directed mutagenesis (QuikChange, Agilent Technologies) using the following primers: for the ΔEDTEE construct, CTTCAGTCCTGGTGGACAGCAGCCGGGGAAG and CTTCCCCGGCTGCTGTCCACCAGGAC TGAAG; for the AATAA construct, GTCCTGGTGGAGCCGCCACTGCAGCGCAGCAGCCG and CGGCTGCTGC GCTGCAGTGGCGGCTCCACCAGGAC; for the EDAEE construct, CTGGTGGAGAGGACGCTGAAGAGCAGCAG and CTGCTGCTCTTCAGCGTCCTCTCCACCAG; for the L350A/I352A construct, GGGGCGATACGCAGCTCCT GAAGCGGAGGCAGCTCGCTGTTG and CAACAGCGAGCTGCCTCCGCTTCAGGAGCTGCGTATCGCCCC; and for the C355S/C356S/C358S construct, GGAGATAGCTCGCAGTAGTTTTAGCTTCAGTCCTGGTGG and CCAC CAGGACTGAAGCTAAAACTACTGCGAGCTATCTCC. The plasmid pcDNA3.1-SERINC5-VN-HA was a gift from Yonghui Zheng (34) and was used for the bimolecular fluorescence complementation (BiFC) assays. We used the mutagenic primers described above to construct pcDNA3.1-SERINC5-ΔEDTEE-VN-HA, pcDNA3.1-SERINC5-ΔEDAEE-VN-HA, and pcDNA3.1-SERINC5-ΔAATAA-VN-HA. The plasmid pcDNA3.1-Nef_{SF2}-V5-VC was a gift from Thomas Smithgall (46). To construct pcDNA3.1-Nef_{NL4-3}-V5-VC or a myristoylation-defective Nef (pcDNA3.1-Nef_{NL4-3} G2A-V5-VC), a 621-bp PCR product bearing NotI and EcoRI restriction sites was generated using template plasmids containing the NL4-3 wild-type or mutant Nef alleles (pCI-NL) (24). The sense PCR primer for the wild type was AGATTCGCGGCCGCACCATGGGTG GCAAGTGGTCAAAAAG, whereas the sense PCR primer for the G2A construct was AGATTCGCGGCCGCA CCATGGCCGGCAAGTGGTCAAAAAG, and the antisense PCR primer was CCGGAGTACTTCAAGAACTGGA ATTCTAAGCA. The purified PCR product and pcDNA3.1-Nef_{SF2}-V5-VC were digested with EcoRI and NotI (New England Biolabs [NEB]), and the DNA was isolated by column purification (Zymo Research). The digested pcDNA3.1-Nef_{SF2}-V5-VC was treated with shrimp alkaline phosphatase (NEB) and then ligated with the PCR products overnight at 16°C and transformed into TOP10 competent cells (Thermo Fisher Scientific). Plasmid DNA was isolated from overnight bacterial cultures, and the sequence was verified via Sanger sequencing. For *in vitro* binding studies, HIV-1 NL4-3 Nef (residues 25 to 206) was fused to either SERINC5 ICL4 or a SERINC5 ICL4 ΔEDTEE mutant using a long, flexible linker. The cDNAs were then cloned into an expression vector, pMAT9s (Addgene plasmid number 112590), using the NcoI and HindIII sites, fusing Nef-SERINC5 ICL4 to maltose-binding protein (MBP-Nef-ICL4). MBP-Nef alone was cloned similarly.

An expression construct for SF2 Nef (pcDNA3.1 vector backbone) was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (deposited by J. Victor Garcia and John Foster) (47, 48). Transmitted-founder (TF) proviral clones CH040 and SUMA were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (from a panel of 10 HIV-1 molecular clones) and deposited by John Kappes and Christina Ochsenbauer (49–53).

NL4-3, SF2, CH040, and SUMA Nef proteins were subcloned into the pcDNA3.1 vector backbone using PCR amplification and restriction digestion, with the addition of a C-terminal FLAG epitope tag. The following PCR primer pairs were used: for NL4-3, sense primer AGATTCGCTAGCATGGGTGGCAAGTGGTC

AAAAAG and antisense primer TGCTTAGAATTCTCACTTATCGTCGTCATCCTTGTAATCGCAGTTCTTGAAGTA CTCCGG; for SF2, sense primer AGATTCGCTAGCATGGGTGGCAAGTGGTCAAAAC and antisense primer TGCTTAGAATTCTCACTTATCGTCGTCATCCTTGTAATCGCAGTCTTTGTAGTACTCCGG; for CH040, sense primer AGATTCGCTAGCATGGGTGGCAAGTGGTCAAAATG and antisense primer TGCTTAGAATTCTCACTT ATCGTCGTCATCCTTGTAATCGCAGTTCTTGTAGTACTCCGGAT; and for SUMA, sense primer AGATTCGCTA GCATGGGTGGCAAGTGGTCAAAAAG and antisense primer TGCTTAGAATTCTCACTTATCGTCGTCATCCTTG TAATCGCAGTCCTTGTAGTACTCCG. Following PCR amplification, the vector backbone and insert were digested using the NheI and EcoRI restriction enzymes and ligated following the above-described protocol. The expression levels of the tagged Nef constructs were tested by Western blotting; all were detectable by using both polyclonal anti-Nef and anti-FLAG antibodies, the patterns of expression were the same using either detection method (data not shown).

Expression, purification, and analysis of GST-SERINC3 loop 10, GST-SERINC5 ICL4, $\mu1_{CTD}$, and $\mu2_{CTD}$ proteins and GST-pulldown assays. We previously reported on the expression, purification, and analysis of GST-SERINC3 loop 10 (a loop analogous to SERINC5 intracellular loop 4) (54). Here, we expressed, purified, and analyzed GST-SERINC5 ICL4 similarly. SERINC5 ICL4 was cloned into the pGEX4T1 vector (GE Life Sciences) with an N-terminal GST tag, and the GST-SERINC5 ICL4 construct was transformed into *E. coli* BL21(DE) cells for protein expression. To express phosphorylated GST-SERINC5 ICL4, the construct was coexpressed with both the α and β subunits of casein kinase II (CK-II). The cells were grown to an optical density at 600 nm (OD_{600}) of ~0.6, induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) overnight at 16°C, and then collected by centrifugation. Cell pellets were lysed using a French press homogenizer. Lysates were clarified by centrifugation at 14,000 rpm. GST-SERINC5 ICL4 was purified using GST-affinity chromatography and HiPrep-Q anion exchange and Superdex 200 size exclusion chromatography. Purified GST-SERINC5 ICL4 coexpressed with CK-II was subjected to liquid chromatography-mass spectrometry (LC-MS) and analyzed as previously reported (54). The C-terminal domains (CTDs) of $\mu1$ ($\mu1_{CTD}$; residues 158 to 423) and $\mu2$ ($\mu2_{CTD}$; residues 159 to 435) were cloned into the pET28a expression vector and His tagged. These constructs were used to transform BL21(DE3) cells for protein expression. The cells were grown to an OD_{600} of ~0.6 to 0.8, induced with 0.5 mM IPTG overnight at 18°C, and then lysed using a French press homogenizer. The cell lysate was clarified by centrifugation at 14,000 rpm. The proteins were purified using an His-Select nickel-affinity gel and HiPrep-S cation exchange and Superdex 200 size exclusion chromatography. The purified GST-SERINC3 loop 10, GST-SERINC5 ICL4, $\mu1_{CTD}$, and $\mu2_{CTD}$ proteins were used for *in vitro* GST-pulldown assays. Equimolar ratios of GST-tagged proteins were mixed with $\mu1_{CTD}$ or $\mu2_{CTD}$, and the mixtures were incubated with GST resin overnight at 4°C. On the next morning, the GST resins were extensively washed with 20 mM Tris-HCl, pH 7.5, to remove unbound proteins. The GST-bound proteins were eluted with 10 mM glutathione reduced in 50 mM Tris-HCl, pH 8.0. The eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining.

Expression of MBP-Nef-ICL4 and GST-$\mu2_{CTD}$-truncated AP-2 and GST-pulldown assays. For protein expression, *E. coli* BL21(DE3) cells were transformed with the MBP-Nef-ICL4 construct or mutant, grown to an OD_{600} of ~0.8, induced with 0.3 mM IPTG, and expressed overnight at 18°C. Cell pellets were harvested by centrifugation and flash-frozen in liquid nitrogen for storage. GST-tagged $\mu2_{CTD}$-truncated AP-2 was prepared as previously described (55): *E. coli* cells overexpressing all four AP-2 subunits were lysed by microfluidization, the cell debris was removed by ultracentrifugation, and the supernatant was applied to Ni-nitrilotriacetic acid (NTA) agarose, followed by application to a glutathione-agarose affinity column (GSTrap HP column; GE Healthcare). The AP-2-containing fractions were pooled, concentrated, and dialyzed into glutathione-free buffer overnight. The MBP-Nef and MBP-Nef-ICL4 proteins were purified by Ni-NTA agarose, anion-exchange chromatography (HiTrap Q column; GE Healthcare) and Superdex 200 size exclusion chromatography. For GST-tagged AP-2 pulldown assays, GST-tagged AP-2 (0.2 mg) and either MBP-tagged Nef or the Nef-ICL4 fusion proteins (0.4 mg, 5-fold molar excess) were mixed in a final volume of 100 μl. The reaction mixtures were loaded onto small, gravity-flow columns containing 0.2 ml glutathione Sepharose 4B resin (GE Healthcare) and incubated for 1 h at 4°C. The protein mixtures and resin were washed extensively with 5× (400 μl) GST binding buffer [50 mM Tris, pH 8.0, 100 mM NaCl, 0.1 mM Tris(2-carboxyethyl)phosphine (TCEP)], and the bound protein complexes were eluted with 4× (200 μl) GST elution buffer containing 10 mM reduced glutathione. The elution fractions were analyzed by SDS-PAGE and stained with Coomassie blue.

Measurement of viral infectivity. Infectivity was measured for virions produced from HEK293 cells, Jurkat TAg cells, or Jurkat TAg *SERINC3/5*-KO cells that had been cotransfected with either an infectious molecular clone of HIV-1 (pNL4-3) or a mutant version harboring a deletion in the *nef* gene (pNL4-3ΔNef) and increasing concentrations of pBJ5-SERINC5-iHA or pBJ5-SERINC5-iHA ΔEDTEE. HEK293 cells were seeded at a density of 5×10^5 cells/ml/well (12-well plates). The cells were transfected on the following day with a total of 1.6 μg plasmid, comprising 1.3 μg pNL4-3 or pNL4-3ΔNef, and increasing concentrations of SERINC5-iHA (as indicated above) or empty plasmid (pBJ5), using the Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions (Thermo Fisher Scientific). For experiments in which Nef or glycoGag was provided in *trans*, HEK293 cells were transfected with a total of 1.6 μg plasmid, comprising 625 ng pNL4-3ΔEnv (Nef positive) or pNL4-3ΔEnvΔNef (Nef negative), 325 ng pVRE (expressing Env) with or without 100 ng Nef-FLAG constructs (NL4-3, SF2, CH040, SUMA), or 100 ng pBJ5-HA-gg189 (glycoGag) and increasing concentrations of SERINC5-iHA (as indicated above) or with an empty plasmid (pBJ5). Cells and supernatants were harvested after 24 h. JTAg *S3/5*-KO cells (3.75×10^5) in 2.5 ml medium were cotransfected (Jurkat-In; MTI Global Stem) with a total of 1.25 μg DNA, comprising 1 μg pNL4-3 or pNL4-3ΔNef, and increasing concentrations of SERINC5-iHA (as indicated above) or with empty plasmid (pBJ5). Cells and supernatants were harvested at 48 h posttransfection. For experiments

comparing the SERINC5 mutants, a single concentration of pBJ5-SERINC5-iHA, pBJ5-SERINC5-HA ΔEDTEE, pBJ5-SERINC5-iHA AATAA, or pBJ5-SERINC5-iHA EDAEE was used (as indicated in the figure legends). Virions were harvested from the supernatants by centrifugation through a 20% sucrose cushion at 23,500 × *g* for 1 h at 4°C. The virus pellet was resuspended in culture medium, and dilutions were used to infect the reporter cell line HeLa P4.R5 in duplicate in a 48-well format. These cells express the HIV-1 coreceptors CD4 and CCR5 and possess a Tat-inducible β-galactosidase gene under the transcriptional control of the HIV-1 long terminal repeat (LTR). At 48 h postinfection, the cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature and then stained with 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overnight. Infectious centers (IC) were imaged and quantified using image analysis software (56). The IC data were normalized to the concentration of the p24 antigen in each viral stock, measured by enzyme-linked immunosorbent assay (ELISA; ABL Bioscience). For experiments evaluating the activity of glycoGag, infectivity was measured using HeLa-TZM-bl cells, which contain a luciferase gene under the transcriptional control of the HIV-1 LTR. HeLa-TZM-bl cells were infected with diluted virus stock in duplicate wells of 96-well plates for 48 h. The culture medium was removed, and the cells were lysed in luciferase reporter gene assay reagent (Britelite; PerkinElmer); luciferase activity was measured, using a luminometer, as relative light units (RLU) and normalized to the p24 concentration. To eliminate the residual Nef phenotype in the absence of transfected SERINC5 expression plasmids, the ratios of the IC assay value/p24 concentration (in nanograms per milliliter) or relative light units (RLU)/concentration of p24 were expressed relative to those for the nontransfected SERINC5 control for each viral genotype, setting values for the nontransfected SERINC5 control to 100% for both the wild type and the ΔNef construct in each experiment.

SERINC5 virion incorporation and Western blot analyses. An aliquot of virions purified as described above was used to measure the virion incorporation of SERINC5. Samples of virions were lysed in 30 μl 1× Laemmli buffer containing 50 mM TCEP [Tris(2-carboxyethyl) phosphine; Sigma] and subjected to standard SDS-PAGE, after adjustment to equal amounts of p24, as measured by ELISA. Cellular samples from all experiments were lysed in extraction buffer (50 mM NaCl, 1% Triton X-100, 50 mM Tris, pH 8.0), nuclei were pelleted by centrifugation, and the total protein concentration of the supernatant was measured by a bicinchoninic acid assay (Thermo Fisher Scientific). Equal protein concentrations were mixed with 2× Laemmli buffer containing 100 mM TCEP. To avoid boiling and the consequent aggregation of SERINC5 (28, 29), the samples were sonicated (Diagenode Bioruptor) before protein separation by SDS-PAGE and Western blotting. The cell lysates and viral pellets were resolved on 10% denaturing SDS-PAGE gels, transferred onto polyvinylidene difluoride (PVDF) membranes, immunoblotted with the antibodies indicated below, and visualized using the Western Clarity detection reagent (Bio-Rad). Chemiluminescence was detected using a ChemiDoc imager system (Bio-Rad). Primary and secondary antibodies were prepared in antibody dilution buffer, consisting of 2% milk in phosphate-buffered saline (PBS) with 0.02% Tween 20 (PBST). The following antibodies were used for detection of the proteins of interest: HA.11 (mouse; BioLegend), β-actin (mouse; Sigma), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; mouse; GeneTex), HIV-1 p24 (mouse; Millipore), and HIV-1 Nef (sheep) (57). The intensities of the protein bands were measured using Bio-Rad ImageLab software. SERINC5 incorporation into virions was quantified and compared to that of p24 as a virion loading control.

Flow cytometry. Surface SERINC5 was measured in HEK293 cells and JTAg *S3/5*-KO cells transfected to express pNL4-3 or pNL4-3ΔNef and pBJ5-SERINC5-iHA or the indicated mutants: the ΔEDTEE, EDAEE, or AATAA mutant. HEK293 cells were transfected with 1.6 μg total plasmid, 100 ng of which was pBJ5-SERINC5-iHA, and JTAg *S3/5*-KO cells were transfected with a total of 1.25 μg plasmid, 250 ng of which was pBJ5-SERINC5-iHA. HEK293 cells were stained at 24 h posttransfection, and JTAg *S3/5*-KO cells were stained at 48 h posttransfection. The cells were then washed with ice-cold fluorescence-activated cell sorting (FACS) buffer (1× PBS, 3% FBS), before staining with mouse anti-HA (diluted 1:200; BioLegend) for 30 min on ice. The cells were pelleted by centrifugation and washed in FACS buffer, before incubation with goat anti-mouse immunoglobulin-Alexa Fluor 647 (diluted 1:200; BioLegend) for 30 min on ice. To detect intracellular p24, the cells were washed in FACS buffer and fixed and permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) and stained with an anti-p24 fluorescein isothiocyanate (FITC) antibody (clone KC57; Beckman Coulter) for 30 min on ice (diluted 1:100 in Perm wash buffer; BD Biosciences). The cells were washed with FACS buffer and PBS, before analysis by flow cytometry. Surface SERINC fluorescence was quantified in at least 1×10^4 p24-positive cells per condition. For the BiFC assays, HEK293 cells were transfected with 0.8 μg of either a single Venus N terminus or Venus C terminus plasmid with an empty vector or pairwise with Venus N- and C-terminal fusion proteins. Relative fluorescence intensity was measured in 1×10^4 cells per condition at 24 h after transfection. Data were collected on a BD Accuri C6 cytometer and analyzed using C-Flow sampler (BD) and FlowJo (v10; FlowJo LLC) software.

Immunofluorescence microscopy. HEK293 cells (2×10^5) or HeLa P4.R5 cells (1.2×10^5) were seeded on poly-D-lysine-coated 12-mm coverslips in 24-well plates at 24 h before transfection. The cells were transfected with a total of 0.8 μg plasmid, comprising 312.5 ng pNL4-3ΔEnvΔNef (Nef negative), 162.5 ng pVRE, 50 ng SERINC5-iHA (WT or ΔEDTEE mutant) with or without 50 ng Nef-FLAG (NL4-3), or empty plasmid (pBJ5). At 24 h posttransfection, the cells were washed in cold PBS and fixed in 4% paraformaldehyde (PFA) in PBS on ice for 5 min and then for 15 min at room temperature (RT). The cells were washed twice in PBS and PFA and then quenched with 50 mM ammonium chloride for 5 min. The cells were permeabilized with 0.2% Triton X-100 for 5 min and blocked with 2% bovine serum albumin (BSA) for 30 min, prior to incubation with fluorescently conjugated primary antibodies, DAPI (4′,6-diamidino-2-phenylindole), mouse anti-FLAG Alexa Fluor 488, and mouse anti-HA Rhx (BioLegend)

diluted 1:400 in 1% BSA in PBS. After 2 h incubation at RT, the cells were washed in PBS and then briefly in water, before mounting in Mowiol (polyvinyl alcohol) mounting medium (prepared in-house). Images were captured at a ×100 magnification (1,344 by 1,024 pixels), using an Olympus IX81 widefield microscope fitted with a Hamamatsu charge-coupled-device camera. For each field, a z-series of images was collected, deconvolved using a nearest-neighbor algorithm (SlideBook software; Imaging Innovations, Inc.), and presented as z-stack projections. Image brightness was adjusted using Adobe Photoshop CS4 software.

Data analysis and presentation. Quantitative analyses were performed as described above. Replicate data sets were combined in Microsoft Excel and GraphPad Prism (v5.0) software. Figures were produced using Adobe Photoshop and Adobe Illustrator (CS3) software.

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