A conserved acidic cluster motif in SERINC5 confers resistance to antagonism by HIV-1 Nef

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Abstract

The cellular protein SERINC5 inhibits the infectivity of diverse retroviruses and is counteracted by the glycoGag protein of MLV, the S2 protein of EIAV, and the Nef protein of HIV-1. Determining 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 highly conserved sequence EDTEE, which is located within a cytoplasmic loop of SERINC5 and is reminiscent of an acidic cluster membrane trafficking signal, increases the sensitivity of SERINC5 to antagonism by Nef while having no effect on the intrinsic activity of the protein as an inhibitor of infectivity. The effects on infectivity correlated with enhanced removal of the ΔEDTEE mutant relative to wild type SERINC5 from the cell surface and with enhanced exclusion of the mutant protein from virions by Nef. Mutational analysis revealed 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 compensatory change in the host antiviral protein as it evolves to escape the effects of the viral antagonist. 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.

Introduction

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 progression to AIDS, despite being non-essential for viral propogation 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). Post-translational 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 down-regulation of the HIV receptor CD4 (6) and the major histocompatibility complex I (MHC-I) from the cell surface (7). To modulate CD4, Nef uses a di-leucine-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 target it to the multivesicular body (MVB) pathway for lysosomal 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 consensus regarding CD4, two mechanisms have been proposed for Nef-mediated modulation of MHC-I: 1) 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 eventual lysosomal degradation (18); and 2) Nef accelerates the internalization of MHC-I from the cell-surface via a PI3-kinase-regulated and ARF6-mediated pathway to promote sequestration of MHC-I within the TGN (19). Either of these mechanisms could lead to a reduction of MHC-I molecules at the cell surface and 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 both for transmission and for 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 di-leucine-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 and 5 (SERINC3 and SERINC5) were identified as such factors; they are transmembrane proteins that incorporate into virions and potently 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 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 inhibits 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, and then stimulating its endocytosis and sending the protein toward lysosomal degradation (34). SERINC5 antagonists have been identified in retroviruses other than HIV and SIV. These include the glycoGag protein of Murine Leukemia Virus (MLV) (35) and the S2 protein of Equine Infectious Amenia Virus (EIAV) (36). While Nef and glycoGag are structurally unrelated, the mechanisms by which they counteract

SERINC5 seem similar: endocystosis 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 protein-level, at least not to the extent observed for other anti-retroviral restriction factors such as *TRIM5α* 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 be a determinant of 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 di-leucine 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: 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 more efficient exclusion of SERINC5 from virions and more efficient downregulation of cell surface SERINC5 by Nef. This enhanced-response phenotype appeared to be specific to Nef; 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 may have specifically evolved to render Nef proteins less active SERINC5-antagonists.

Materials and Methods

Cells: HEK293 (obtained from Dr. Saswati Chaterjee) (42) and HeLa TZM-bl cells (obtained from Dr. John Kappes via the NIH AIDS Reagent Program) were cultured in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin. HeLa P4.R5 cells (obtained from Dr. Ned Landau) were maintained in DMEM media supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 1 µg/ml puromycin. A leukemic T cell clone (Jurkat E6.1) lacking endogenous levels of SERINC3 and 5 (termed JTAg *S3/5* KO) was a gift from Dr. Heinrich Gottlinger. These cells were cultured in complete RPMI media: 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 *env* and *nef* genes ("DHIVΔNef") were gifts from Dr. 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 *Xho*I site in Nef (100 bp downstream of the Nef start codon), and encodes 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 Dr. Massimo Pizzato. The plasmid pBJ5-SERINC5-iHA was a gift from Dr. Heinrich Gottlinger (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 and pBJ5-SERINC5-iHA EDAEE, containing mutations within amino acids 364-368 in human SERINC5, were generated using site-directed mutagenesis (QuikChange, Agilent Technologies) using the following primers: for ΔEDTEE:

163 CTTCAGTCCTGGTGGACAGCAGCCGGGGAAG and CTTCCCCGGCTGCTGTCC-
164 ACCAGGACTGAAG; for AATAA: GTCCTGGTGGAGCCGCCACTGCAGCG-
165 CAGCAGCCG and CGGCTGCTGCGCTGCAGTGGCGGCTCCACCAGGAC; for
166 EDAEE: CTGGTGGAGAGGACGCTGAAGAGCAGCAG and CTGCTGCTCTT-
167 CAGCGTCCTCTCCACCAG. The plasmid pcDNA3.1-SERINC5-VN-HA was a gift from
168 Dr. Yonghui Zheng (34) and was used for the Bi-molecular Fluorescence
169 Complementation (BiFC) assays. We used the mutagenic primers above to construct
170 pcDNA3.1-SERINC5ΔEDTEE-VN-HA, pcDNA3.1-SERINC5 EDAEE-VN-HA and
171 pcDNA3.1-SERINC5 AATAA-VN-HA. The plasmid pcDNA3.1-Nef$_{SF2}$-V5-VC was a gift
172 from Dr. Thomas Smithgall and has been described previously (46). To construct
173 pcDNA3.1-Nef$_{NL43}$-V5-VC or a myristoylation defective Nef (pcDNA3.1-Nef$_{NL43}$ G2A-V5-
174 VC), a 621 bp PCR product bearing *Not*I and *Eco*RI restriction sites was generated
175 using template plasmids containing the NL4-3 wildtype or mutant Nef alleles (pCI-NL)
176 (24). The sense PCR primer for wildtype was AGATTCGCGGCCGC-
177 ACCATGGGTGGCAAGTGGTCAAAAAG, whereas the sense PCR primer for G2A was
178 AGATTCGCGGCCGCACCATGGCCGGCAAGT-GGTCAAAAAG; the antisense PCR
179 primer was CCGGAGTACTTCAAGAACTGGAATTCTAAGCA. The purified PCR
180 product and pcDNA3.1-Nef$_{SF2}$-V5-VC were digested with *Eco*RI and *Not*I (NEB), and
181 the DNA was isolated by column purification (Zymo Research). The digested
182 pcDNA3.1-Nef$_{SF2}$V5-VC was treated with shrimp alkaline phosphatase (NEB), then
183 ligated with the PCR products overnight at 16°C and transformed into TOP10
184 competent cells (Thermo Fisher Scientific). Plasmid DNA was isolated from overnight
185 bacterial cultures and verified via Sanger sequencing. For *in vitro* binding studies, HIV-

1 NL4-3 Nef (residues 25 to 206) was fused to either SERINC5 intracellular loop 4 (ICL4; residues 332 to 387) or a SERINC5 ICL4 ΔEDTEE mutant using a long, flexible linker. The cDNAs were then cloned into an expression vector pMAT9s (Addgene Plasmid # 112590) using the *NcoI* and *HindIII* sites, fusing SERINC5 Nef-ICL4 to maltose binding protein (MBP-Nef-ICL4). MBP-Nef alone was cloned similarly.

Expression, purification,and analysis of GST-SERINC3-Loop10, GST-SERINC5-ICL4, MBP-μ1 and MBP-μ2 proteins: We previously reported expression, purification, and analysis of GST-SERINC3-Loop10 (a loop analogous to SERINC5 intracellular loop 4) (47). 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 co-expressed with both the α and β-subunits of Casein Kinase II (CK-II). The cells were grown to OD600 of ~0.6, induced with 0.1mM IPTG overnight at 16°C, 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, HiPrep-Q anion exchange and S200pG chromatography. Purfied GST-SERINC5-ICL4 co-expressed with CK-II was subjected to LC/MS and analyzed as previously reported (47). For the expression of MBP-μ1 and MBP-μ2, we used previously described constructs (48) encoding truncated versions of μ1 (residues 158-423) and μ2 (residues 159-435). These proteins were co-expressed with the pGro7 chaperone in BL21 cells, which was induced by 1.5g/L L-(+)-arabinose at OD600~0.2. To express MBP-μ1 and MBP-μ2, the cells were

209 further grown to OD600~0.6 and induced with 0.1mM IPTG overnight at 16°C. Cells
210 were lysed using a French press homogenizer. The cell lysate was clarified by
211 centrifugation at 14,000 RPM. The proteins were purified using His-Select Nickel affinity
212 gel, Hi Prep-S cation exchange and S200pg gel-filtration chromatography.
213 *Recombinant Protein Expression for Nef-SERINC5-AP2 pulldowns*: For protein
214 expression, *E. coli* BL21(DE3) cells were transformed with the MBP-Nef-ICL4 construct
215 or mutant, grown to OD_{600} ~ 0.8, induced with 0.3 mM IPTG, and expressed overnight at
216 18°C. Cell pellets were harvested by centrifugation and flash-frozen in liquid nitrogen for
217 storage. GST-tagged $\mu2_{CTD}$-truncated AP-2 was prepared as previously described (49):
218 *E.coli* cells overexpressing all four AP-2 subunits were lysed by microfluidization, cell-
219 debris removed by ultracentrifugation, and the supernatant applied to Ni-NTA agarose
220 followed by glutathione-agarose affinity column (GSTrap HP, GE Healthcare). AP-2-
221 containing fractions were pooled, concentrated, and dialyzed into glutathione-free buffer
222 overnight. MBP-Nef and MBP-Nef-ICL4 proteins were purified by Ni-NTA agarose,
223 anion exchange chromatography (HiTrap Q, GE Healthcare), and Superdex 200 size
224 exclusion chromatography.
225 *GST-pulldowns using recombinant proteins.* Purified, GST-SERINC3-Loop10, GST-
226 SERINC5-ICL4, MBP-μ1 and MBP-μ2 proteins were used for *in vitro* GST-pulldowns.
227 An equimolar ratio of GST-tagged proteins were mixed with MBP-μ1 or MBP-μ2, and
228 these mixtures were incubated with GST-resin overnight at 4°C. The next morning, the
229 GST-resins were extensively washed with 20mM Tris-HCL pH 7.5 to remove unbound
230 proteins. The GST-bound proteins were eluted with 10mM glutathione reduced in 50mM
231 Tris HCL pH 8.0. The eluted fractions were analyzed by SDS-PAGE using Coomassie

Blue stain. 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. Reaction mixtures were loaded onto small, gravity flow columns containing 0.2 mL glutathione Sepharose 4B resin (GE Healthcare), and incubated for 1 hour at 4°C. Protein mixtures and resin were washed extensively with 5x (400 µL) GST binding buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM TCEP), and bound protein complexes were eluted with 4x (200 µL) GST elution buffer containing 10 mM reduced glutathione. Elution fractions were analyzed by SDS-PAGE and stained with Coomassie Blue.

Measurement of Viral Infectivity: Infectivity was measured in virions produced from HEK293 cells or Jurkat TAg *SERINC3/5* KO cells co-transfected 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 x 10^5 cells/ml/well (12-well plates). The cells were transfected 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), or empty plasmid (pBJ5), using Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions (Thermo Fisher Scientific). For experiments including glycoGag (Figure 8), HEK293 cells were transfected with a total of 1.6 µg plasmid; 625 ng pNL4-3ΔEnv (Nef+) pNL4-3ΔEnvΔNef (Nef-), 325 ng pVRE (expressing Env), with or without 100 ng pBJ5-HA-gg189 (glycoGag), and increasing concentrations of SERINC5-iHA (as indicated), or empty plasmid (pBJ5). Cells and supernates were harvested after 24 hr. 3.75 x 10^5

JTAg S3/5 KO cells in 2.5ml medium were co-transfected (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), or empty plasmid (pBJ5). Cells and supernates were harvested 48 hr post-transfection. For experiments comparing 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 supernates 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 used to infect the reporter cell line HeLa P4.R5 in duplicate in a 48-well format. These cells express the HIV-1 co-receptors CD4 and CCR5 and possess a Tat-inducible β-galactosidase gene under the transcriptional control of the HIV-1 LTR. 48 hr post-infection, 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 (50). The IC data were normalized to the concentration of p24 antigen in each viral stock measured by 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 hours. The culture medium was removed, and the cells were lysed in luciferase reporter gene assay reagent (Britelite, Perkin Elmer); luciferase activity was measured using a

luminometer as relative light units (RLU), and normalized to the p24 concentration. To eliminate residual Nef-phenotype in the absence of transfected SERINC5-expression plasmids, the IC/ng p24 or relative light units RLU/ng p24 were expressed relative to the no-transfected-SERINC5 control for each viral genotype, setting the no-transfected-SERINC5 values to 100% for both wildtype and ΔNef in each experiment.

SERINC5 Virion Incorporation and Western blots: An aliquot of virions purified as described above was used to measure virion-incorporation of SERINC5. The samples of virions were lysed in 30 µl 1x 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 (50mM NaCl, 1% Triton-X-100, 50mM Tris, pH 8.0), nuclei pelleted by centrifugation, and total protein concentration of supernatant was measured by BCA assay (Thermo Fisher Scientific). Equal protein concentrations were mixed with 2x Laemmli buffer containing 100 mM TCEP. To avoid boiling and 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 indicated antibodies, and visualized using 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 PBST (PBS with 0.02% Tween 20). The following antibodies were used for

detection of proteins of interest: HA.11 (mouse, Biolegend), β-actin (mouse, Sigma), GAPDH (mouse, Genetex), HIV-1 p24 (mouse, Millipore) and HIV-1 Nef (sheep) (51).

Flow Cytometry: Surface SERINC5 was measured in HEK293 cellls and JTAg S3/5 KO cells transfected to express pNL4-3 or pNL4-3ΔNef and pBJ5-SERINC5-iHA or the indicated mutants: ΔEDTEE, EDAEE or AATAA. 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, of which 250 ng was pBJ5-SERINC5-iHA. HEK293 cells were stained 24 hours post-transfection and JTAg S3/5 KO cells 48 hours post-transfection. The cells were then washed with ice-cold FACS Buffer (1x PBS + 3% FBS) before staining with mouse anti-HA (diluted 1:200, Biolegend) for 30 minutes on ice. The cells were pelleted by centrifugation and washed in FACS buffer before incubation with goat anti-mouse Alexa Fluor 647 (diluted 1:200, Biolegend) for 30 minutes 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 FITC antibody (clone KC57; Beckman Coulter) for 30 minutes 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 x 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 or Venus-C plasmid with an empty vector, or pairwise with Venus N- and C- terminal fusion proteins. Relative fluorescence intensity was measured in 1 x 10^4 cells per condition, 24 hours after transfection. Data were collected on a BD Accuri C6 Cytometer and analyzed using C-Flow sampler (BD) and FlowJo (v10, FlowJo LLC) Software.

Data Analysis and Presentation:

Quantitative analyses were performed as described above. Replicate datasets were combined in Microsoft Excel and Graphpad Prism 5.0 software. Figures were produced using Adobe Photoshop and Adobe Illustrator (CS3) software.

Results

The EDTEE sequence reduces the sensitivity of SERINC5 to HIV-1 Nef.

An alignment between human and various primate SERINC5 proteins revealed a highly conserved acidic sequence (EDTEE; Figure 1) located in a long, predicted cytoplasmic loop (designated intra-cytoplasmic loop 4 (ICL4)). The EDTEE sequence is reminiscent of an acidic cluster membrane trafficking signal, therefore we hypothesized that it might be a Nef-response sequence and support Nef-activity. To test this, we co-transfected HEK293 cells or Jurkat TAg cells that lack endogenous SERINC3 and 5 (*SERINC* 3/5 KO) with pNL4-3 (an HIV-1 infectious molecular clone) or its Nef-negative counterpart (pNL4-3 ΔNef) along with increasing amounts of plasmids expressing either SERINC5-HA (pBJ5-SERINC5-iHA) or SERINC5-HA ΔEDTEE (pBJ5-SERINC5-iHA ΔEDTEE). The virions produced were partially purified by centrifugation through a 20% sucose cushion, and their infectivity was measured using an infectious center (IC) assay. The IC values were divided by the concentration of p24 capsid antigen, and the IC/p24 ratios were normalized to the "no-added SERINC5" control, setting that control value to 100% for both the wild type 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

346 SERINC5 or related mutants by transfection. For our HEK293 cells, the *nef*-infectivity-
347 phenotype in the absence of plasmid-mediated expression of SERINC5 was 6- to10-fold
348 and was presumably due to the endogenous expression of SERINC family members
349 (data not shown). For the Jurkat TAg *SERINC* 3/5 KO cells, the *nef*-infectivity-
350 phenotype in the absence of plasmid-mediated expression of SERINC5 was
351 approximately 2-fold, despite the genetic disruption of both *SERINC* family members
352 with known anti-viral activity (data not shown).

353 As expected, we observed a dose dependent antiviral effect of SERINC5, which
354 was greater when virions were produced in the absence of Nef (Figure 2A).
355 Surprisingly, deletion of the EDTEE sequence enhanced the sensitivity of SERINC5 to
356 Nef, but it did not affect the inhibitory activity of the protein in the absence of Nef (Figure
357 2A). The difference in sensitivity to Nef was not clearly attributable to differences in
358 SERINC5 protein expression within viral producer cells (Figure 2B, which shows a
359 representative experiment in which Jurkat cells were used to produce virions). However,
360 a subtle influence of the deletion on protein-expression was apparent in the dose-
361 response western blot data: the ΔEDTEE mutant seemed slightly underexpressed in the
362 absence of Nef, although it was expressed equivalently to wild type SERINC5 in the
363 presence of Nef. Consistent with the infectivity data, deletion of the EDTEE sequence
364 caused substantially enhanced exclusion of SERINC5 from virions by Nef (Figure 2C,
365 which again shows a representative experiment in which Jurkat cells were used to
366 produce virions). Notably, we confirmed that a 55 kDa form of SERINC5, while the
367 minority species in cells, is the predominant form in virions, an effect due to the
368 selective incorporation into virions of a form of the protein modified by complex glycans

(52). Here, the data suggested that the ΔEDTEE mutant might be slightly less efficiently incorporated into virions than wild type SERINC5 in the absence of Nef, although the difference is subtle. Overall, these data support the current model that Nef-mediated exclusion of SERINC5 from virions correlates with enhanced infectivity (28, 29). The data further 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.

The EDTEE sequence is phosphorylated by casein kinase II in vitro but does not interact with the μ subunit of AP-2.

We reported recently that a phosphoserine acidic cluster (PSAC) motif of sequence SGASDEED is present in a cytoplasmic loop of 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 that the serines of this sequence are under positive selection (38, 47). The SGASDEED sequence of SERINC3 has potential as a membrane sorting or trafficking sequence, however, because it binds the medium (μ) subunits of AP-1 (μ1) and AP-2 (μ2) in a serine-phosphorylation dependent manner (47). Here we observed that when the recombinant SERINC5 loop containing the EDTEE sequence (ICL4) was co-expressed as a GST-fusion protein together with casein kinase II in *E.coli*, the threonine of the EDTEE sequence, as well as upstream serines in the loop, were phosphorylated (Figure 3A). Nonetheless, unlike the analogous loop of SERINC3, phosphorylated SERINC5 ICL4 did not bind to recombinant μ2 *in vitro* (Figure 3B). These data indicate that although the

391 EDTEE sequence has the potential for threonine-phosphorylation, *in vitro* binding data
392 do not support a role as a μ-binding clathrin-adaptor sorting signal.
393
394 *The acidic residues within the EDTEE motif but not the threonine affect Nef-sensitivity.*
395 We next sought to determine whether the acidic nature of the SERINC5 EDTEE
396 motif is the determinant of sensitivity to Nef. We created SERINC5 mutants that either
397 lacked (EDTEE mutated to AATAA) or preserved (EDTEE mutated to EDAEE) acidic
398 residues and tested their restrictive activity and sensitivity to Nef. SERINC5-AATAA,
399 but not SERINC5-EDAEE, was characterized by a relatively enhanced sensitivity to Nef
400 that was similar to the phenotype of SERINC5-ΔEDTEE in both HEK293 and JTAg *S3/5*
401 KO cells (Figure 4A). All the SERINC5 mutants were as restrictive as the wild type
402 protein; that is, they inhibited the infectivity of virions produced in the absence of Nef as
403 effectively as wild type SERINC5, despite that the expression of the ΔEDTEE and
404 AATAA mutants (but not the EDAEE mutant) seemed slightly reduced. Overall, these
405 data suggest that the relative acidity or negative charge of the SERINC5 EDTEE region
406 affects sensitivity to Nef but not intrinsic restrictive activity. The data also indicate that
407 the threonine alone is not a substantial determinant of sensitivity to Nef, even though it
408 would contribute to the negative charge of the region if phosphorylated.
409
410 *Deletion of the EDTEE sequence enhances Nef's ability to downregulate SERINC5.*
411 Nef co-opts endocytic machinery, namely AP-2 and clathrin, to downregulate
412 SERINC5 from the plasma membrane (28, 29). We reasoned based on our virologic
413 data that the ability of Nef to downregulate SERINC5 would be increased by deletion of

414 the EDTEE sequence. To study the downregulation of cell surface SERINC5 by Nef, we
415 co-transfected cells with pNL43 or pNL43ΔNef together with the SERINC5-iHA
416 expression-plasmids and measured surface SERINC5 levels by immunofluorescent
417 staining and flow cytometry. All of the SERINC5 constructs (wild type, ΔEDTEE,
418 AATAA, and EDAEE) were similarly expressed at the cell surface in Jurkat TAg
419 *SERINC3/5* KO cells in the absence of Nef (Figure 5A, lower panel). SERINC5-
420 ΔEDTEE and SERINC5-AATAA, but not SERINC5-EDAEE, were downregulated more
421 efficienty than wild type SERINC5 in both JTAg *S3/5* KO and HEK293 cells (Figure 5).
422 These results are consistent with the virologic data and support the correlation between
423 the downregulation of cell surface SERINC5 and the enhancement of infectivity by Nef.
424 The data further support that the EDTEE sequence provides relative resistance to Nef-
425 mediated modulation of SERINC5.
426
427 *Deletion of the SERINC5 EDTEE sequence does not enhance interaction with Nef.*
428 We used a recently reported bimolecular fluorescence complementation (BiFC)
429 assay (34, 46) to test the hypothesis that the interaction of Nef and SERINC5 is
430 enhanced in the absence of the EDTEE sequence. In this assay, the two proteins of
431 interest are fused to either the N- or C-terminus of Venus (yellow fluoresecent protein).
432 A fluorescent signal is generated if the two proteins interact, enabling the quantitative
433 measurement of protein-protein interactions within living cells. Here, we fused the N-
434 terminus of Venus to the C-terminus of either wild-type NL4-3 Nef or a myristolyation-
435 signal mutant incapable of associating with membranes (Nef G2A) (6). We also fused
436 the C-terminus of Venus to the C-terminus of either SERINC5, SERINC5ΔEDTEE,

437 SERINC5 EDAEE or SERINC5 AATAA. These constructs were used to transfect
438 HEK293 cells either singly or in pairs, and the relative fluorescence was measured by
439 flow cytometry twenty-four hours later. We detected modest fluorescence when only
440 Nef-VN or SERINC5-VC was expressed and a 4-fold relative increase in fluorescence
441 when these two proteins were co-expressed (Figure 6A). This increase of the
442 fluorescent signal was lost when SERINC5-VC was paired with Nef-G2A-VN, consistent
443 with the notion that Nef requires membrane-association to interact with SERINC5. We
444 did not detect an increased interaction-signal when Nef-VN was paired with either
445 SERINC5-ΔEDTEE-VC or SERINC5-AATAA-VC relative to SERINC5-VC (Figure 6A).
446 No differences in the expression of these fusion proteins was detected by western blot
447 (Figure 6B). These data suggest that deletion of the SERINC5 EDTEE sequence does
448 not enhance its interaction with Nef.
449
450 *Deletion of the EDTEE sequence does not enhance binding of a Nef-SERINC5*
451 *cytoplasmic loop fusion protein to AP-2* in vitro.
452 We next sought to determine whether deletion of the EDTEE sequence
453 enhances formation of a ternary complex including Nef, a cytoplasmic loop of SERINC5
454 and AP-2. As noted above, a previous study showed that the long cytoplasmic loop
455 within SERINC5 - ICL4 - confers Nef-responsiveness (39). Because ICL4 contains the
456 EDTEE sequence, we produced recombinant proteins containing NL4-3 Nef (residues
457 25-206) fused via a long flexible linker to either SERINC5 ICL4 (residues 332-387) or a
458 SERINC5 ICL4 ΔEDTEE mutant (Figure 7A). A protein containing only the Nef
459 component served as a control. Each of these proteins was fused to maltose-binding-

460 protein (MBP) to enhance their solubility. Binding of these proteins to a recombinant,
461 $\mu2_{CTD}$-truncated AP-2 heterotetramer *in vitro* was analyzed by pulldown assays using
462 GST-tagged AP-2 mixed wth either MBP-Nef, MBP-Nef-SERINC5 ICL4 or MBP-Nef
463 SERINC5 ICL4 ΔEDTEE (Figure 7B). ICL4 strikingly stimulated the pulldown of Nef with
464 the $\mu2_{CTD}$-truncated AP-2 complex, a result consistent with the notion that this
465 cytoplasmic loop is a Nef-response sequence and that Nef and ICL4 together bind
466 efficiently to AP-2. However, we detected little or no influence of the EDTEE sequence
467 in this assay: the Nef-ICL4 fusion protein did not clearly bind more efficiently to AP-2
468 when the EDTEE sequence was deleted (Figure 7B). These results suggest that the the
469 EDTEE sequence does not interfere with formation of a Nef, SERINC5-ICL4, AP-2
470 complex when assessed using recombinant proteins *in vitro.*
471
472 *Role of the EDTEE sequence in the antagonism of SERINC5 by glycoGag.*
473 Based on our results with Nef, we hypothesized that the EDTEE sequence might
474 affect the antagonism of SERINC5 by the glycosylated Gag (glycoGag) protein of
475 Moloney murine leukemia virus (M-MLV). MLV glycoGag counteracts SERINC3 and 5
476 and rescues infectivity of *nef*-deficient HIV-1 (35). The majority of the extracellular
477 domain of M-MLV glycoGag is dispensable for this activity (53). We therefore used a
478 minimal active truncated form of glycoGag which contains the N-terminal 189 residues
479 (gg189) to test the ability of glycoGag to rescue the infectivity of HIV-1 lacking Nef
480 in the presence of either SERINC5 or the SERINC5-mutant lacking the EDTEE
481 sequence. For these experiments, HIV-1 Env was provided in *trans* in the virion-
482 producer cells, in order to abrogate syncytia-formation in the target cells, which was

483 strikingly exaggerated when the absence of Nef was complimented by glycoGag
484 and Env was encoded in the viral genome (data not shown). HeLa-TZM-bl indicator
485 cells were used for luminometric measurement of infectivity, as this provided a more
486 sensitive method for measuring the infectivity of the pseudo-virions. We confirmed that,
487 as shown above, the EDTEE sequence provided relative resistance to Nef when
488 infectivity was measured using this modified assay design (Figure 8). Moreover, as
489 reported previously, glycoGag efficiently antagonized the activity of SERINC5 as an
490 inhibitor of infectivity (Figure 8). Unlike Nef, however, the activity of glycoGag was
491 not enhanced when the EDTEE sequence of SERINC5 was deleted. In contrast, the
492 activity of glycoGag against the EDTEE-mutant was slightly diminished. These data
493 suggest that the role of the EDTEE sequence in SERINC5 is Nef-specific, despite
494 that the cellular cofactors involved in SERINC antagonism by Nef and glycoGag
495 appear to be similar (28, 29, 53).
496
497 **Discussion**
498 We intially hypothesized that the acidic cluster within the long cytoplasmic loop of
499 SERINC5 - the sequence EDTEE in ICL4 - might function as a protein sorting motif in
500 concert with HIV-1 Nef, and thus support Nef-activity as a SERINC5-antagonist.
501 Instead, our data indicate that Nef is more effective as a SERINC5-antagonist in the
502 absence of the EDTEE sequence. A SERINC5 mutant lacking this sequence, or a
503 mutant in which the acidic residues are replaced with alanines (but not a mutant in
504 threonine is replaced with alanine), is more effectively antagonized by Nef at the levels
505 of counteraction of SERINC5-mediated inhibition of infectivity, down-regulation of

SERINC5 from the cell surface, and exclusion of SERINC5 from virions. In general, these data are consistent with the current model of surface down-regulation and virion-exclusion of SERINC5 as the basis for Nef-mediated enhancement of infectivity, and they support the notion that the region of SERINC5 containing the EDTEE sequence determines Nef-sensitivity.

While our work was in progress, Dai and colleagues mapped the intracellular cytoplasmic loop that contains the EDTEE sequence (designated ICL4 in their study) as the key region of the protein required for sensitivity to Nef (39). These investigators identified two hydrophobic residues in the N-terminal half of the loop that are required for response to Nef. While our data are consistent with the conclusion that ICL4 contains determinants of Nef-sensitivity, they reveal that in addition to residues that support Nef-activity, the loop also contains a sequence - EDTEE- that is inhibitory to Nef-activity.

Why would a cytoplasmic loop of SERINC5 contain such an inhibitory sequence? 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 potentially 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.

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. However, we found no evidence that deletion of the EDTEE sequence influences the subcellular localization of SERINC5 (data not shown), nor does the loop containing the EDTEE sequence bind the μ subunit of the clathrin adaptor AP-2 *in vitro*, as the analogous loop of SERINC3 does.

Another possibility is that the negative charge of the EDTEE sequence inhibits the 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 bi-molecular fluorescence complementation did not support this model: the interaction 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 expression of the ΔEDTEE and AATAA mutants is sufficient to increase their apparent Nef-responsiveness. Several lines of evidence weigh against this possibility. First, the SERINC5 mutants appear to reach the plasma membrane, the presumed site of Nef

552 counteraction via endocytosis, as efficiently as the wild type protein (as measured by
553 flow cytometry). Second, none of our data suggest that the intrinsic restrictive activities
554 of the mutant proteins are decreased, weighing against the functional significance of the
555 subtle differences detected in some or our western blots. Third, and perhaps most
556 importantly, the ΔEDTEE mutant was not more responsive than the wild type SERINC5
557 to glycoGag; rather, the mutant appeared slightly less responsive. The observation that
558 the effect of deleting the EDTEE sequence is opposite when testing responsiveness to
559 Nef versus glycoGag is inconsistent with the notion that the observed virologic
560 phenotypes are consequences of the levels of protein expression.

561 These considerations leave open the question of exactly how the EDTEE motif in
562 SERINC5 specifically inhibits the activity of Nef as a SERINC5-antagonist. Although our
563 binding experiments using recombinant proteins *in vitro* do not clearly support the
564 hypothesis that the EDTEE sequence inhibits the interaction between Nef-SERINC5
565 and the AP-2 clathrin adaptor complex, we have not yet attempted to assess this
566 ternary interaction in the more complex environment of human cells. A structural
567 explanation of the interaction between Nef, SERINC5 ICL4, and AP-2 might yet provide
568 an answer for the currently enigmatic role of this motif.

569

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Figure Legends

Figure 1: An acidic cluster motif (EDTEE) is highly conserved within human and non-human primate SERINC5. A.) Predicted topology of SERINC5 showing ten transmembrane domains and six cytoplasmic domains, four of which form loops. The EDTEE sequence is shown in red and is found within the long cytoplasmic loop-4 (ICL-4). B.) Amino-acid sequence alignment of SERINC5 ICL-4. The human sequence, the sequences of several non-human primates, and the murine sequence are shown. The conserved EDTEE acidic cluster motif is shown in red.

Figure 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 5 (JTAg *S3/5* KO) were transfected to express NL4-3 (WT) or a *nef*-negative mutant (NL4-3ΔNef) and increasing doses of SERINC5-HA or a mutant lacking the acidic cluster motif (SERINC5-iHAΔEDTEE), 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 infectious centers (IC) imaged and quantified. The IC/ml were divided by the concentration (ng/ml) of p24 antigen measured in the virion-preparations by ELISA. The infectious centers per nanogram (IC/ng) were normalized to the no-added-SERINC5 control for each viral genotype (wild type: "+ Nef" or Nef-negative: "- Nef". Data are presented as the mean percentage relative infectivity, error bars are the standard deviation (s.d.) from *n=2* (HEK293 cells)

772 and *n=3* (JTAg *S3/5* KO cells) experiments. B.) Protein from whole JTAg *S3/5* KO cell
773 lysates from the experiment of panel A were subjected to SDS-PAGE and Western
774 blotting. Membranes were probed with antibodies to detect SERINC5 (HA), Nef, p24,
775 and β-actin. C.) Virions produced by JTAg *S3/5* KO cells and analyzed in the
776 experiment of panel A were normalized by p24 content before SDS-PAGE. Membranes
777 were probed for p24/55, Nef, and SERINC5 (HA). Nef is cleaved within virions by the
778 viral protease, yielding a 16 kDa C-terminal product (51).
779
780 **Figure 3: Phosphorylated SERINC5-ICL4 does not bind directly to the medium (μ)**
781 **subunit of AP-2.** A) Sequence of SERINC5-ICL4. GST-SERINC5-ICL4 was co-
782 expressed with casein kinase II (CK-II) in *E.coli*, purified, and analyzed by liquid
783 chromatography/mass spectrometry (LC/MS). Phospho-peptide sequences are shown,
784 and sites of serine-threonine (ST) phosphorylation are highlighted in red and the residue
785 locations are indicated. Mascot scores for the peptide matches are shown. B) Serinc5-
786 ICL4 does not bind to μ2. GST-SERINC3 loop 10 (a positive control) and GST-
787 SERINC5-ICL4 fusion proteins were expressed in *E. coli* either with or without CK-II and
788 tested for binding to μ2 in GST-pulldown assays. The μ2 protein is N-terminally
789 truncated and fused to maltose binding protein (MBP) as a solubility tag. SDS/PAGE
790 gels were stained with Coomassie Blue.
791
792 **Figure 4: The acidic residues within the SERINC5 EDTEE sequence, but not the**
793 **threonine, are important for resistance to Nef.** A.) HEK293 cells or JTAg *S3/5* KO
794 cells were transfected with pNL4-3 or pNL4-3ΔNef and indicated amounts of plasmid

795 expressing either WT SERINC5 or the following mutants: ΔEDTEE, EDAEE, or AATAA.
796 Virions were harvested and infectivity assays performed in HeLa P4.R5 cells, as
797 described in Figure 2. The infectious centers per nanogram (IC/ng) were normalized to
798 the no-added-SERINC5 control for each viral genotype. Data are presented as the
799 mean percentage relative infectivity, error bars are the s.d. from *n=2* (HEK293 cells) and
800 *n=3* (JTAg *S3/5* KO cells) experiments. B.) Protein derived from whole JTAg *S3/5* KO
801 cell lysates from the experiment of A were subjected to SDS-PAGE and Western
802 blotting. Membranes were probed with antibodies to detect SERINC5 (HA), Nef, p24/55,
803 and GAPDH.
804
805 **Figure 5: Mutation of the EDTEE sequence enhances the downregulation of**
806 **SERINC5 from the cell surface by Nef**. JTAg *S3/5* KO or HEK293 cells were
807 transfected with pNL4-3 (+ Nef) or pNL4-3ΔNef (- Nef) and either 250 ng (JTAg *S3/5*
808 KO) or 100ng (HEK293) of plasmid expressing WT SERINC5 or the EDTEE mutants:
809 ΔEDTEE, EDAEE, or AATAA. The cells were stained for surface SERINC5 (HA, Alexa
810 Fluor-647) and intracellular p24 (FITC). A.) Representative two-color dot-plots showing
811 surface expression of SERINC5 (HA) in p24-positive JTAg *S3/5* KO cells (+/- Nef). B.)
812 The MFI of surface SERINC5 (HA) in p24-positive cells was quantified +/- Nef. Data are
813 presented as mean percentage MFI normalized to no-Nef control, error bars are the s.d.
814 of *n=2* (JTAg *S3/5* KO cells) or *n=4* (HEK293 cells) experiments.
815
816 **Figure 6: Intracellular interaction between Nef and SERINC5 as measured by**
817 **bimolecular fluorescence complementation (BiFC) is not substantially affected by**

818 **the EDTEE sequence.** A.) HEK293 cells were transfected with plasmid constructs
819 expressing Nef-Venus-C (VC), Nef G2A-VC, SERINC5-Venus-N (VN),
820 SERINC5ΔEDTEE-VN, SERINC5 EDAEE-VN or SERINC5 AATAA-VN either singly or
821 in the pairs indicated. Twenty-four hours later, the fluorescence intensity (FL1) was
822 measured by flow cytometry. The data are presented as the mean fluorescence
823 intensity of Venus signal, error bars are s.d. for *n=3* independent experiments. B.)
824 Protein from whole cell lysates from a representative experiment was subjected to SDS-
825 PAGE and Western Blotting. Membranes were probed with anti-V5 (Nef-VC), HA
826 (SERINC5-VN) or GAPDH (loading control) antibodies.
827
828 **Figure 7: SERINC5 (ICL4) stimulates the interaction of Nef with AP-2 *in vitro***
829 **independently of the EDTEE sequence.** A.) Schematic of recombinant protein
830 constructs used to study formation of a Nef-SERINC5 ICL4 complex *in vitro*. A
831 heterotetrameric AP-2 complex core with a GST tag on the α subunit; the C-terminal
832 two-thirds of the μ2 subunit is deleted, as are the appendage domains of the α and β2
833 subunits. Nef was fused to SERINC5 ICL4 (with or without the EDTEE sequence) via a
834 linker peptide; these proteins were further fused to the maltose-binding-protein (MBP)
835 as a solubility tag (not shown). MBP-Nef alone was used as a control. B.) GST-
836 pulldown assay assessing the binding of Nef-SERINC ICL4 (with or without the EDTEE
837 sequence) or Nef alone to the truncated AP2 core *in vitro*. The input protein mixtures,
838 the protein(s) washed through the GST-matrix, and the protein(s) that remained bound
839 to the GST-matrix were run on an SDS/PAGE gel and stained with Coomassie Blue.
840

Figure 8: Deletion of the EDTEE sequence does not enhance the activity of MLV Glycogag as an antagonist of SERINC5. A.) HEK293 cells were co-transfected with plasmid constructs encoding 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 minimal active glycoGag (HA-gg-189) was also provided in *trans*. Virions were harvested twenty-four hours post-transfection, partially purified by centrifugation through a sucrose cushion, and used to infect HeLa-TZM-bl luciferase indicator cells. Luciferase activity was measured 48 hr post-infection and normalized to p24 antigen (relative light units, RLU/ng p24). Data are expressed as percentage infectivity relative to the no added SERINC control for each condition (with or without Nef or glycoGag). Error bars indicate standard deviation for *n=2* independent experiments. (B) Comparison of relative infectivity of virions in absence or 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. Membranes were probed with antibodies to detect SERINC5 (HA), glycoGag (gg189, HA), Nef, p55 (Gag), and GAPDH.

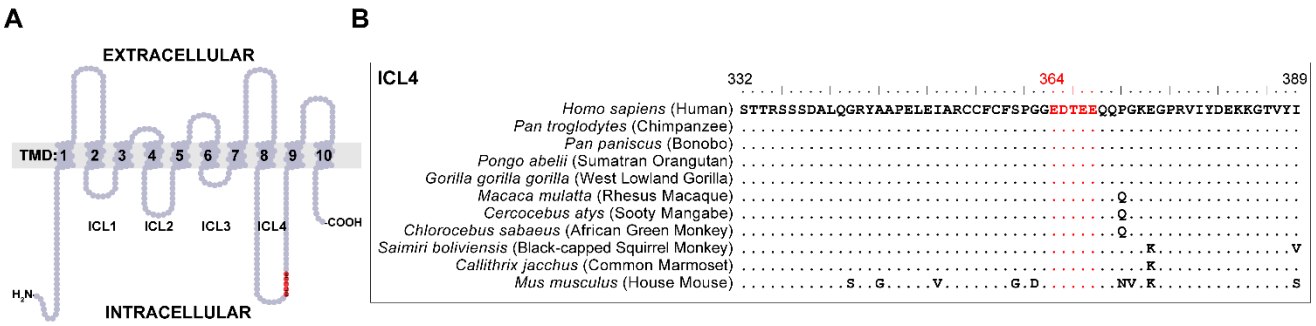


FIGURE 1

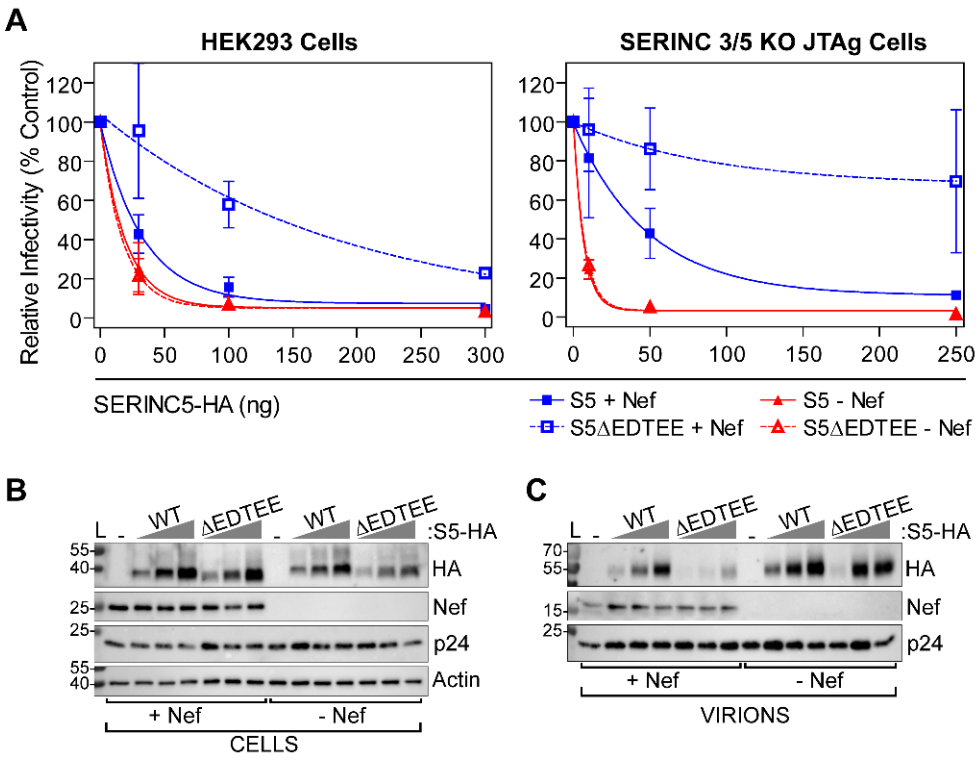


FIGURE 2

A

SERINC5 ICL4:

$_{332}$**STTRSSSDALQGRYAAPELEIARCCFCFSPGGEDTEEQQPGKEGPRVIYDEKKGTVYI**$_{389}$

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

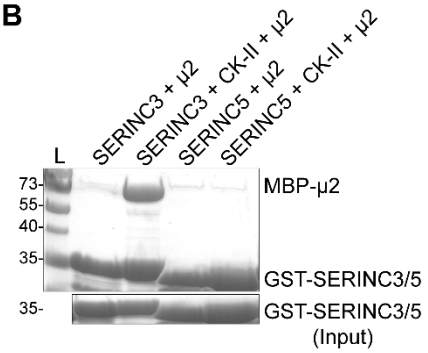
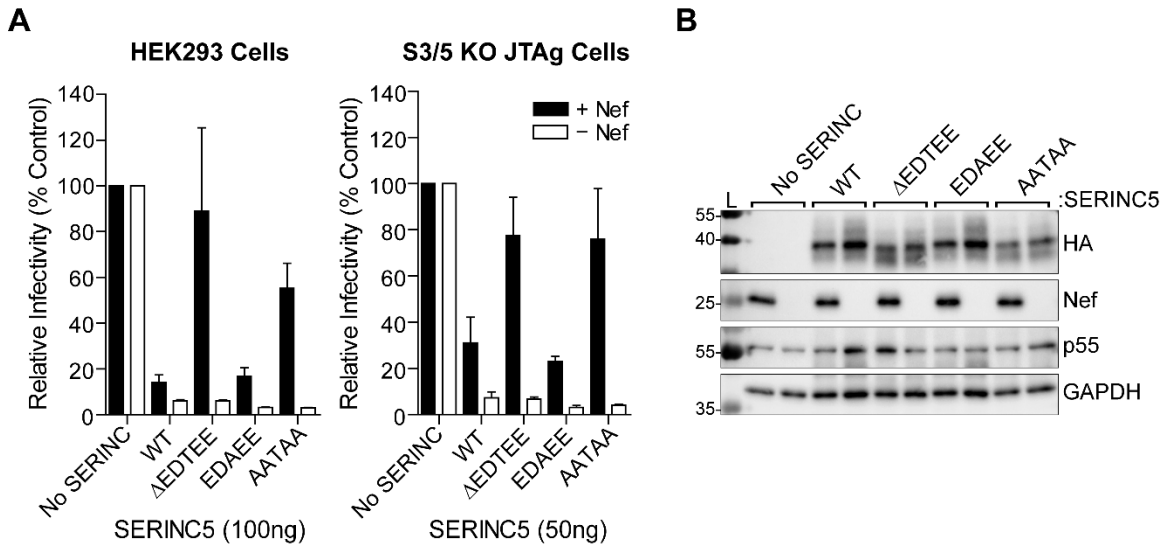


FIGURE 3



871

872

873 **FIGURE 4**

874

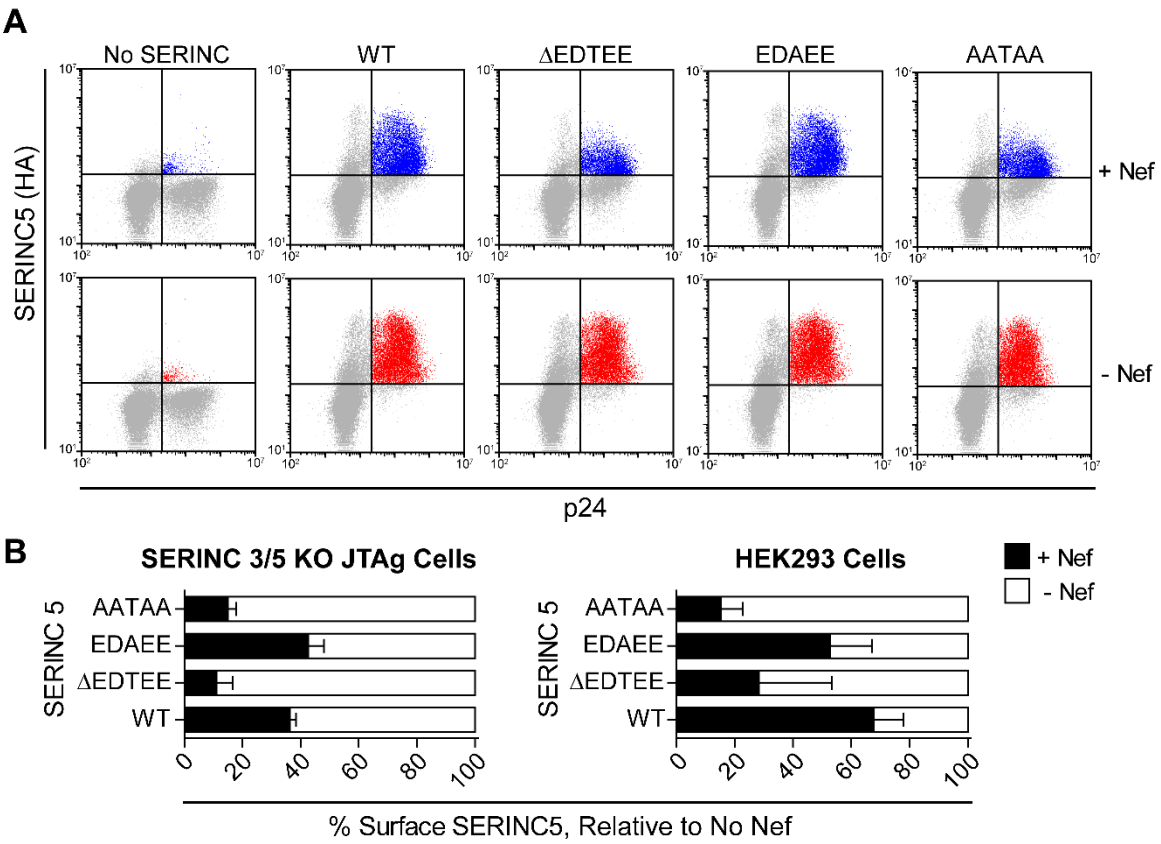
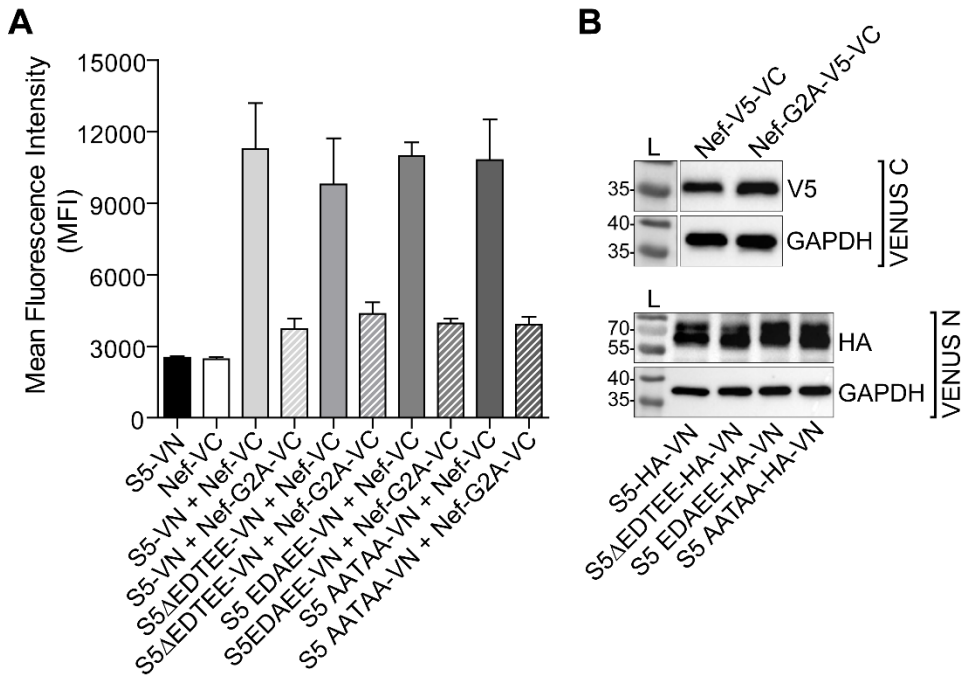


FIGURE 5



879

880 **FIGURE 6**

881

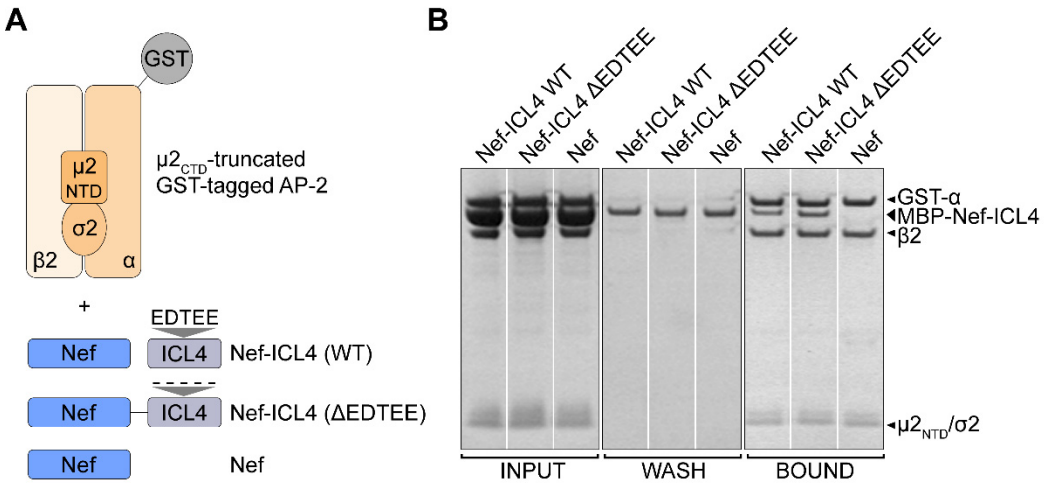


FIGURE 7

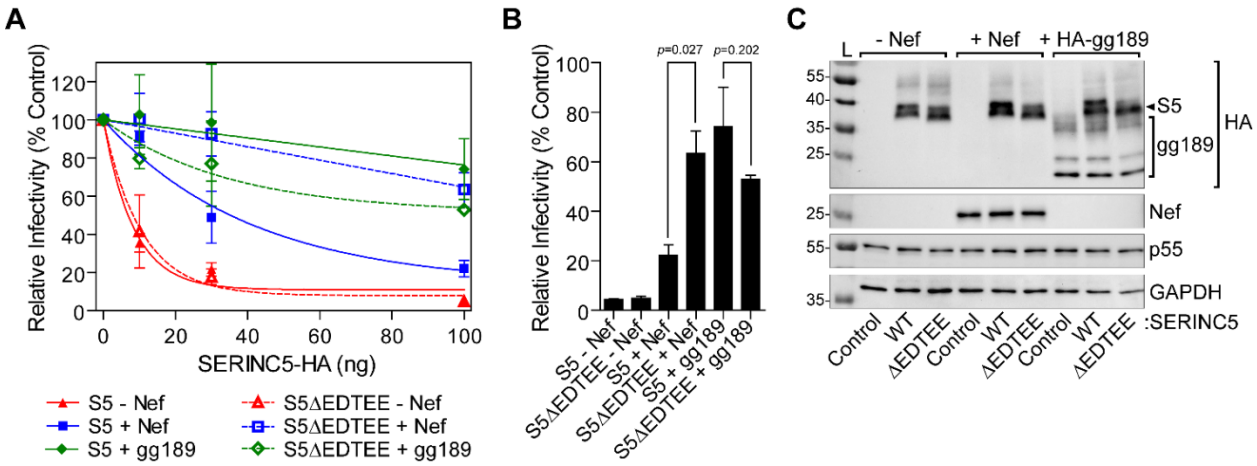


FIGURE 8