S2 from equine infectious anemia virus is an infectivity factor which counteracts the retroviral inhibitors SERINC5 and SERINC3

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The lentivirus equine infectious anemia virus (EIAV) encodes the small protein S2, a pathogenic determinant that is important for virus replication and disease progression in horses. No molecular function had been linked to this accessory protein. We report that S2 can replace the activity of Negative factor (Nef) in HIV-1 infectivity, being required to antagonize the inhibitory activity of Serine incorporator (SERINC) proteins on Nef-defective HIV-1. Like Nef, S2 excludes SERINC5 from virus particles and requires an ExxxLL motif predicted to recruit the clathrin adaptor, Adaptor protein 2 (AP2). Accordingly, functional endocytic machinery is essential for S2-mediated infectivity enhancement, and S2-mediated enhancement is impaired by inhibitors of clathrin-mediated endocytosis. In addition to retargeting SERINC5 to a late endosomal compartment, S2 promotes host factor degradation. Emphasizing the similarity with Nef, we show that S2 is myristoylated, and, as is compatible with a crucial role in posttranslational modification, its N-terminal glycine is required for anti-SERINC5 activity. EIAV-derived vectors devoid of S2 are less susceptible than HIV-1 to the inhibitory effect of both human and equine SERINC5. We then identified the envelope glycoprotein of EIAV as a determinant that also modulates retroviral susceptibility to SERINC5, indicating that EIAV has a bimodal ability to counteract the host factor. S2 shares no sequence homology with other retroviral factors known to counteract SERINC5. Like the primate lentivirus Nef and the gammaretrovirus glycoGag, the accessory protein from EIAV is an example of a retroviral virulence determinant that independently evolved SERINC5-antagonizing activity. SERINC5 therefore plays a critical role in the interaction of the host with diverse retrovirus pathogens.

retrovirus | restriction factor | virus infection

During the past 30 y several cellular factors known as "restriction factors" have been identified that are capable of inhibiting different steps in the retrovirus life cycle and signaling an ongoing pathogen invasion (1). However retroviruses have evolved the ability to overcome such cellular inhibitors either by escaping recognition by these factors or by actively antagonizing them. Some retroviruses count on increased genome plasticity and exploit alternative splicing to encode accessory proteins to antagonize intrinsic host defenses (2). Negative factor (Nef) from primate lentiviruses and glycosylated Group antigens (Gag) protein (glycoGag) from gammaretroviruses exert convergent activity (3) on the infectivity of HIV and murine leukemia virus (MLV) by counteracting the cellular multipass transmembrane proteins Serine incorporator 5 (SERINC5) and 3 (SERINC3) (4, 5). SERINC5 inhibits retrovirus infection of target cells at an early stage, following its incorporation into virions. Both Nef and glycoGag antagonize SERINC5 by promoting its intracellular relocalization via a clathrin-dependent mechanism and preventing its incorporation into virions. Human SERINC5 inhibits the divergent retroviruses HIV-1 and MLV efficiently, indicating low species-specific barriers. Given that Nef and glycoGag have evolved the ability to counteract SERINC5 in primate lentiviruses and gammaretroviruses independently, we sought to investigate whether the selective pressure imposed by this host factor has affected the evolution of other retroviruses. Because SERINC5 is highly expressed in blood-derived cells, we investigated whether another blood-tropic retrovirus, Equine infectious anemia virus (EIAV), also evolved a Nef-like infectivity factor. EIAV is a myeloid-tropic lentivirus that causes anemia and thrombocytopenia in horses worldwide and chronicizes into an inapparent, asymptomatic carrier stage. EIAV is a complex retrovirus that expresses the 7-KDa auxiliary protein S2 from doubly spliced mRNA (6). S2 has no homology to other known proteins, and its molecular activities remain unknown. Intriguingly, in vivo mutant EIAV lacking a functional S2 ORF phenotypically resembles Nef-defective HIV-1. Although there is no evidence that S2 is required for EIAV replication in vitro, its requirement as a pathogenic factor in vivo has been well established, because infection of animals with a virus lacking a functional S2 is associated with low viral load and the absence of clinical symptoms (7–9).

Here we report that S2 has evolved the ability to antagonize SERINC5 and its homolog SERINC3, indicating that, as do Nef and glycoGag, the recently discovered host restriction factor plays a crucial role in the biology of retroviruses and has shaped their evolution.

Significance

Serine incorporators (SERINC) 5 and 3 are recently discovered cellular inhibitors of retroviruses, which are incorporated into virus particles and impair their ability to propagate the infection to target cells. Only two groups of viruses (represented by HIV-1 and murine leukemia virus) had been identified as having evolved the ability to counteract SERINC inhibition. We found that equine infectious anemia virus, which causes a debilitating disease in horses, also has acquired the capacity to protect the virus particle from inhibition by SERINC5 and SERINC3 by using its small protein S2. That three different retroviruses have independently evolved the ability to elude inhibition by SERINC5 and SERINC3 indicates that these cellular factors play a fundamental role against various retrovirus pathogens.

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Results

S2 Rescues the Infectivity of Nef-Defective HIV-1. We first investigated whether S2 could functionally replace the activity of Nef in HIV-1 infectivity. The S2 ORF cloned from the pSPEIAV19 strain (10) was inserted into an *env*-defective HIV-1_{NL4-3} molecular clone in place of *nef*. The resulting construct was used to produce HIV-1 limited to a single replication cycle by transfecting JTAg cells. HIV-1 encoding S2 was sixfold more infectious than *nef*-defective HIV-1_{NL4-3}, indicating that S2 can restore most of the infectivity lost in the absence of Nef (Fig. 1*A*). To monitor the expression of S2 by Western blotting and immunofluorescence, the S2 coding sequence was optimized based on human codon use and was fused to an HA tag. Although the N-terminally tagged protein was poorly detected by Western blotting and was inactive with respect to HIV-1 infectivity, the HA tag at the C terminus was detected, and the protein's activity was similar to that of the untagged protein expressed from the native ORF (Fig. S1). Therefore S2-HA was used in subsequent experiments. When S2-HA was expressed *in trans*, it restored the infectivity of Nef-defective HIV-1 to a level similar to that of the WT counterpart (Fig. 1*B*), but it did not significantly alter HIV-1 infectivity in the presence of Nef, indicating that the activity of S2 and Nef are complementary rather than additive. The ability of S2 to restore the infectivity of Nef-defective HIV-1 was confirmed further using a different S2 allele derived from the highly pathogenic EIAV strain Wyoming (Fig. S2).

The effect of Nef on HIV-1 infectivity has distinctive features because it depends on the envelope (env) glycoprotein and the producer cell type (4). Therefore we investigated whether the effect of S2 has the same features. As established previously, HIV-1 produced from CEMX174 cells does not require Nef to produce optimally infectious virus. Similarly, S2 had no effect on the infectivity of HIV-1 derived from CEMX174 cells (Fig. 1*C*). HIV-1 pseudotyped with vesicular stomatitis virus G-protein (VSV-G) or with the env glycoprotein derived from HIV-1_{JRFL} is not responsive to Nef (Fig. 1*D*). Similarly, S2 expression in JTAg producer cells did not alter the infectivity of Nef-defective HIV-1 progeny virus pseudotyped with VSV-G or HIV-1_{JR-FL} env (Fig. 1*D*). Nef and S2 therefore are similar in their dependence on cell type and env, further highlighting a functional similarity.

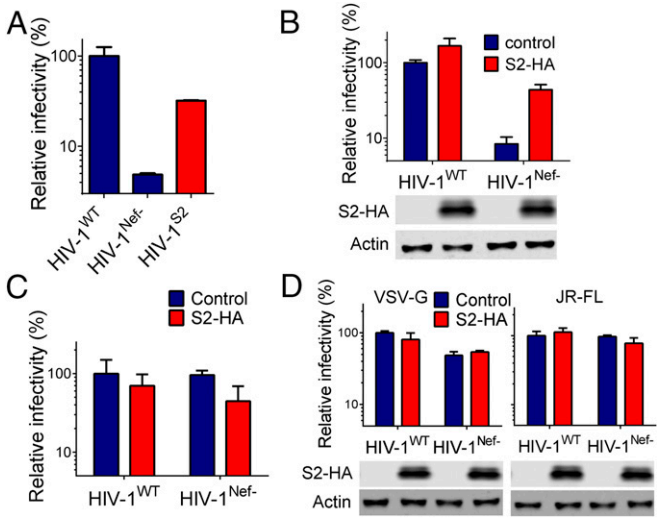

Fig. 1. EIAV S2 and HIV-1 Nef are functionally similar infectivity-promoting factors. (*A* and *B*) S2 repairs the defective infectivity of Nef-defective single-cycle HIV-1_{NL4-3} produced in JTAg cells. S2 was expressed in place of Nef within the HIV-1_{NL4-3} provirus (*A*) or *in trans*, fused to an HA tag at the C terminus (*B*). (*C* and *D*) S2 and Nef have the same effects on infectivity because S2 does not affect the infectivity of HIV-1_{NL4-3} derived from CEMx174 (*C*) or of HIV-1_{NL4-3} pseudotyped with VSV-G or JR-FL env (*D*). Relative infectivity is expressed as the percent of the infectivity of WT HIV-1_{NL4-3} in the absence of S2 expression. Error bars represent the SD of the mean calculated from quadruplicate determinations. *B* and *D* include Western blot detection of S2-HA and β-actin in lysates of virus-producing cells.

S2 Counteracts SERINC5 and SERINC3. We next investigated whether S2, as does Nef, acts by antagonizing SERINC5 and SERINC3.

To do so, we first investigated whether S2 requires both SERINC5 and SERINC3 to increase HIV-1 infectivity in JTAg cells. To assess the importance of both SERINC genes, Nef-defective HIV-1 was produced from WT JTAg cells, from JTAg cells lacking SERINC5 (JTAg$^{SERINC5\ KO}$) (4), or from JTAg$^{SERINC5\ KO}$ cells cotransfected with CRISPR/Cas9 constructs targeting the SERINC3 coding sequence, as previously reported (JTag$^{SERINC5/3\ KO}$) (4). Nef-defective HIV-1 is characterized by 18-fold lower infectivity than virus derived from JTag$^{SERINC5/3\ KO}$ cells and sixfold lower infectivity than virus derived from JTag$^{SERINC5\ KO}$ cells (Fig. 2*A*). As is compatible with the ability to counteract both restriction factors, the expression of S2 resulted in a 10-fold increase in the infectivity of HIV-1 produced from WT cells and only a threefold increase of the infectivity of virus derived from JTag$^{SERINC5\ KO}$ cells. In contrast, S2 did not alter the infectivity of HIV-1 derived from JTag$^{SERINC5/3\ KO}$ cells (Fig. 2*A*). Paralleling the effect of S2, Nef increased HIV-1 infectivity 12-fold, indicating that both retroviral accessory proteins enhance HIV-1 infectivity similarly by counteracting SERINC5 and SERINC3.

To confirm further the ability of S2 to antagonize SERINC5 and SERINC3, the host factors were ectopically expressed in HEK293T cells during virus production. Increasing amounts of SERINC5-expressing plasmid inhibited Nef-defective HIV-1 infectivity by two- to 50-fold in the absence of S2-HA (Fig. 2*B*). In contrast, virus infectivity remained unchanged when S2 was coexpressed in producer cells, irrespective of the amount of SERINC5-HA plasmid transfected. Similarly, the expression of SERINC3-HA resulted in a twofold inhibition of Nef-defective HIV-1 infectivity; this inhibition was rescued completely by S2 expression (Fig. S3).

Therefore the EIAV accessory protein S2 promotes virus infectivity by counteracting SERINC5 and SERINC3.

S2 Prevents SERINC5 and SERINC3 Incorporation into Virus Particles. We previously established that SERINC5 is incorporated efficiently into retroviral particles and that HIV-1 Nef and MLV glycoGag act in producer cells by promoting the exclusion of SERINC5 from virions. We investigated whether S2 acts in a similar way. Nef-defective HIV-1 particles were produced in HEK293T cells transfected to express HIV-1, SERINC5-HA, and S2-HA. As revealed by Western blotting, although SERINC5-HA was readily detected in Nef-defective virions, the expression of S2-HA drastically reduced its incorporation, because the protein was detected at a level similar to background (Fig. 3*A*). Similarly, S2-HA also prevented the incorporation of SERINC3-HA (Fig. 3*B*). Therefore S2, as do Nef and glycoGag, acts by excluding the host factors from virus particles.

We observed that the steady-state level of SERINC5 in the lysate of virus-producing cells was reduced when S2 was expressed (Fig. 3*A*), indicating that S2 may affect SERINC5-HA stability. To investigate this possibility further, we tested the effect of S2 with an increasing amount of SERINC5-HA–expressing plasmid and a constant amount of S2-HA–expressing vector in HEK293T cells. S2 coexpression caused decreased SERINC5-HA levels in cell lysates, irrespective of the amount of plasmid transfected (Fig. 3*C*). However, contrary to the effect observed on SERINC5-HA, the steady-state level of FLAG-tagged CXCR4, another multipass transmembrane protein, was not affected by S2-HA (Fig. 3*D*). This result indicates that the S2 effect on the steady-state level of SERINC5 is selective.

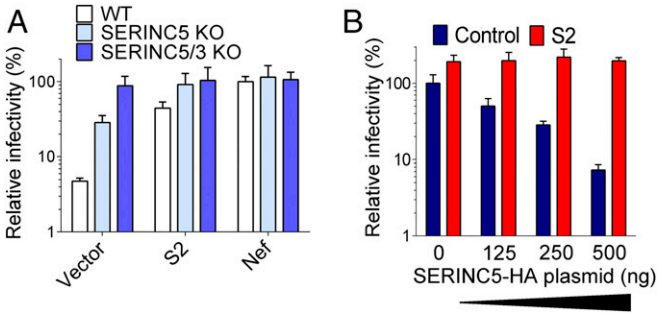

Fig. 2. S2 counteracts the inhibition of HIV-1 by SERINC5 and SERINC3. (*A*) The effect of S2 on the infectivity of Nef-defective HIV-1_{NL4-3} produced in JTAg cells requires endogenously expressed SERINC5 and SERINC3. Shown is the infectivity of Nef-defective HIV-1^{NL4-3} produced in JTAg cells knocked out for SERINC5 or knocked out for both SERINC5 and SERINC3. (*B*) S2 preserves the infectivity of Nef-defective HIV-1_{NL4-3} from inhibition by PBJ6-SERINC5-HA transfected in producer HEK293T cells. Relative infectivity is expressed as the percent of the infectivity of Nef-defective HIV-1_{NL4-3} in the absence of S2 expression. Error bars represent the SD of the mean calculated from quadruplicate determinations.

S2 Requires Clathrin-Dependent Vesicular Trafficking and a Conserved Putative Di-Leucine Sorting Motif to Counteract SERINC5. Both Nef and glycoGag cause a dramatic relocalization of SERINC5 from the plasma membrane into a perinuclear Ras-related protein 7 (Rab7)-positive late endosomal compartment (4). We investigated whether S2 acts in a similar way. When SERINC5-GFP was expressed by transfecting JTAg cells, together with a control protein (RFP), its localization was restricted almost exclusively to the plasma membrane (Fig. 4*A*). In contrast, when a plasmid encoding S2-HA was cotransfected in place of RFP, the host factor readily relocalized into a perinuclear compartment and only partly colocalized with S2-HA. Cotransfection with a plasmid expressing Rab7-RFP revealed that the accumulation of SERINC5-GFP by S2 occurs in a compartment that is labeled with red fluorescence (Fig. 4*B*). This finding indicates that, similarly to Nef and glycoGag, S2 expression results in the relocalization of SERINC5 from the cell surface to the late endosomal compartment, suggesting the involvement of endocytosis.

Therefore we investigated whether the formation of clathrin-mediated coated pits affected the ability of S2 to counteract SERINC5. Vectors encoding transdominant-negative dynamin 2 (Dyn2K44A), which inhibits vesicle constriction, and the C-terminal fragment of the clathrin adaptor AP180 (AP180C), which interferes more specifically with clathrin-dependent invagination of the cell surface (11), were found to impair the ability of the EIAV accessory protein to promote progeny virus infectivity (Fig. 4*C*), indicating that S2 engages the intravesicular trafficking machinery and requires clathrin-mediated endocytosis to counteract SERINC5.

The S2 amino acid sequence contains conserved putative motifs that also can be observed in Nef (6–9, 12). These motifs include a possible myristoylation signal and an SH3-binding motif (PxxP). We also have observed the presence of either an ExxxLL or a YxxL putative Adaptor protein 2 (AP2)-binding motif (Fig. 4*D*) similar to those found in Nef and glycoGag (13, 14). To verify whether ExxxLL is required for S2's effect on infectivity, the two leucine residues were mutated to alanine, and the ability of the resulting mutant protein to counteract SERINC5 was tested. Although the double mutation did not alter the expression level of the protein (Fig. 4*E*), it abolished its ability to promote viral infectivity in the presence of SERINC5, indicating that the ExxxLL motif plays an important role in the activity of S2. In contrast, the putative SH3-binding motif PxxP is dispensable for the activity of S2 on SERINC5 (Fig. S4).

S2 Is Myristoylated. Both Nef and glycoGag associate with the plasma membrane, because Nef is N-terminally myristoylated and glycoGag is an integral type II transmembrane protein. It has been suggested previously that S2 could be myristoylated, as is Nef (6, 12, 15), based on the presence of a possible canonical myristoylation signal including Gly^7 and Ser^{11} located five residues from the N terminus (Fig. 4*D*). However, experimental evidence confirming this possibility has never been reported. Because myristoylation always occurs at the N-terminal glycine, we investigated the importance of Gly^2 for the ability of S2 to counteract SERINC5. An S2 mutant protein in which the N-terminal glycine was replaced with alanine (S2 G2A) was generated for this purpose. Mutant S2 G2A-HA was expressed in HEK293T cells producing HIV-1 in the presence of SERINC5-HA. Although WT S2-HA rescued Nef-defective HIV-1 infectivity in the presence of SERINC5 (Fig. 5*A*), the mutant protein failed to counteract the host factor despite being detected in cell lysates at a similar level as the WT protein (Fig. 5*A*). The N-terminal glycine therefore plays a crucial role in S2's ability to counteract SERINC5, as is consistent with the possible involvement of myristoylation.

To assess directly in vivo whether S2 can be myristoylated, cells expressing S2-HA were labeled with azide-conjugated myristic acid, and the presence of S2-HA in the azide-labeled enriched fraction of cell lysates was probed by Western blotting using an anti-HA antibody. WT S2-HA (Fig. 5*B*) was readily detected in the enriched fraction, whereas only a signal indistinguishable from background was detected in samples expressing S2-HA lacking the N-terminal glycine (S2 G2A). Similarly, Nef-HA, but not Nef G2A, was readily visible in the enriched fractions, whereas a nonmyristoylated control protein (CypA-HA) produced no signal above background. These results indicate that EIAV S2 can be specifically targeted by the myristoyl transferase.

N-terminal myristoylation is instrumental for association with cellular membranes. Accordingly, as shown in Fig. 4*A*, in transfected JTAg cells WT S2-HA accumulates in vesicular structures, partly colocalizing with SERINC5-GFP, and at the cell periphery (Fig. 5*C*). The G2A S2 mutant, which failed to relocalize SERINC5, instead was distributed more diffusely throughout the cytoplasm. These results suggest that the N-terminal glycine is crucial for S2's intracellular localization with vesicles and with

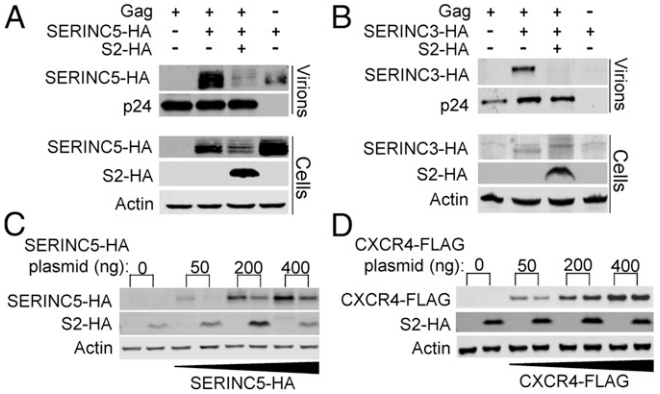

Fig. 3. S2 prevents virion incorporation of SERINC5 and SERINC3. (*A* and *B*) S2 expression in HEK293T cells producing Nef-defective HIV-1_{NL4-3} prevents virion incorporation of SERINC5-HA (*A*) and SERINC3-HA (*B*). (*C* and *D*) Western blotting of virion pellets and lysates of the respective producer cells. S2-HA alters the steady-state expression level of SERINC5-HA (*C*) but not CXCR4-FLAG (*D*). Shown are Western blots of lysates from HEK293T cells that were cotransfected with PBJ5 plasmid encoding S2-HA and increasing amounts of SERINC5-HA (*C*) or CXCR4-FLAG (*D*) expressed from PCDNA vectors.

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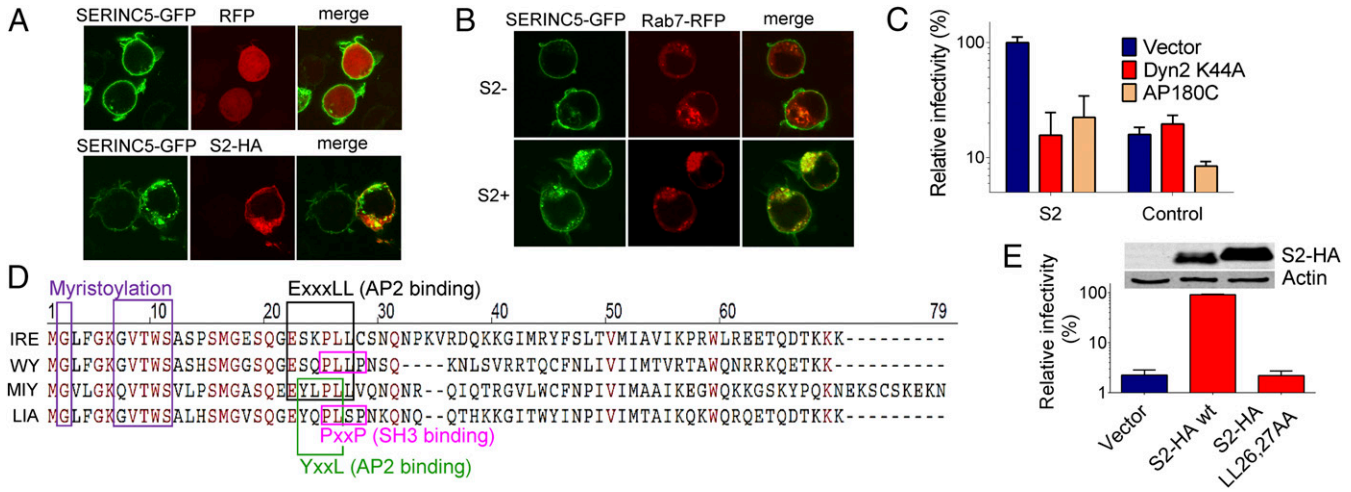

Fig. 4. Counteraction of SERINC5 by S2 requires clathrin-dependent endocytosis and a conserved putative AP2-binding motif. (*A* and *B*) S2 causes the relocalization of SERINC5-GFP from the plasma membrane to a Rab7-labeled compartment. Shown is fluorescence confocal imaging of JTAg cells transfected to express the indicated proteins. (*C*) The ability of S2 expressed *in trans* to counteract SERINC5 is impaired by the clathrin-dependent endocytosis inhibitors Dyn2K44A and AP180-C in HEK293T cells transfected to produce Nef-defective HIV-1_{NL4-3}. (*D*) ClustalW alignment of S2 amino acid sequences derived from four isolates representing the four major EIAV groups: $EIAV_{wy}$ (WY; GenBank accession no. AAC03764), $EIAV_{IRE}$ (IRE; GenBank accession no. AFW99166), $EIAV_{LIA}$ (LIA; GenBank accession no. AAK21109), and $EIAV_{MIY}$ (MIY; GenBank accession no. AFV61766). Putative functional motifs are boxed and color-coded. (*E*) The integrity of the ExxxLL sequence is required for S2 to counteract the activity of SERINC5 on Nef-defective HIV-1_{NL4-3}. Virus was produced by transfecting HEK293T cells expressing WT or mutant S2-HA. Western blotting shows the expression of S2-HA and β-actin in lysates of producer cells. Infectivity in *C* and *E* is expressed as a percentage relative to Nef-defective HIV-1_{NL4-3} produced in the presence of WT S2-HA and the absence of inhibitors. Error bars represent the SD of the mean calculated from quadruplicate determinations.

the plasma membrane and that myristoylation has a role in S2's effect on infectivity.

Sensitivity to SERINC5 Is also Modulated by EIAV Env. After using HIV-1 to establish S2's ability to counteract SERINC5, we also investigated the role of S2 in the context of EIAV retrovirus particles.

Recombinant EIAV vector particles were produced by transfecting HEK293T cells ectopically expressing human SERINC5-HA. The infectivity of progeny recombinant particles was quantified by transducing TZM-bl cells engineered to express equine ELR1, the EIAV receptor, stably (16). In the absence of S2, EIAV vector particles carrying an EIAV env were inhibited by SERINC5 only threefold (Fig. 6*A*, *Left*), indicating that EIAV retrovirus particles are less sensitive to the host factor than HIV-1 (Fig. 6*A*, *Right*). However, in the presence of S2, SERINC5 did not affect the infectivity of EIAV vectors, confirming the ability of the accessory protein to counteract the inhibition exerted by the host factor.

Compared with the human protein, the equine SERINC5 molecule has additional 83 amino acids at the N terminus (Fig. S5). We therefore investigated whether the magnitude of EIAV inhibition by SERINC5 is species specific and whether the equine retrovirus would be more sensitive to equine SERINC5. When human and equine SERINC5 were expressed in HEK293T cells producing EIAV vector particles, a similar magnitude of inhibition was observed irrespective of the ortholog used (Fig. 6*B*), thus ruling out a species-specific antiretroviral activity. Therefore the EIAV vectors used are intrinsically less sensitive to SERINC5, irrespective of S2 expression.

Env glycoprotein is a crucial determinant of the susceptibility of retrovirus particles to SERINC5 (Fig. 1*D*). Therefore we investigated whether the lower sensitivity of EIAV virions to SERINC5 is a feature associated with the viral core or depends on the env glycoprotein.

To discriminate between these two possibilities, we investigated the ability of SERINC5 to inhibit EIAV pseudotypes. After unsuccessfully attempting to generate infectious EIAV pseudotypes bearing HIV-1 env, we used MLV-A env instead to study EIAV vector particles carrying an env glycoprotein known to render HIV-1 fully sensitive to SERINC5 (4). Expression of either human or equine SERINC5 in producer cells resulted in a 10- to 20-fold reduction in the infectivity of the EIAV/MLV-A hybrid particles (Fig. 6*C*), indicating that when pseudotyped with an

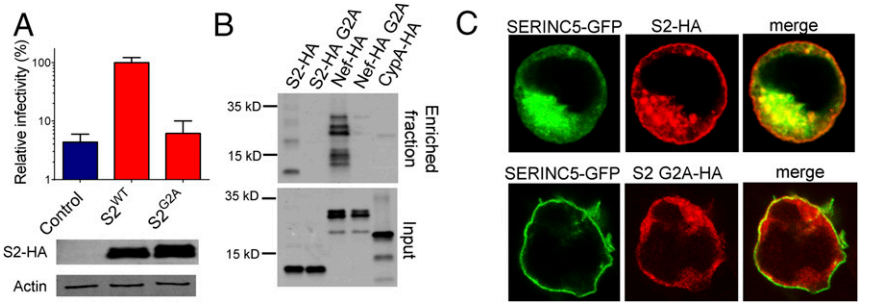

Fig. 5. The role of N-terminal glycine for S2 activity. (*A*) Gly^2 is required for SERINC to counteract S2-HA on Nef-defective HIV-1_{NL4-3}. The virus was produced by transfecting HEK293T cells to express WT or mutant S2-HA. Western blotting shows the expression of S2-HA and β-actin in lysates of producer cells. Infectivity is expressed as the percentage relative to the infectivity of Nef-defective HIV-1_{NL4-3} produced in the presence of WT S2-HA. Error bars represent the SD of the mean calculated from quadruplicate determinations. (*B*) S2 is myristoylated. Shown is Western blotting to detect HA-tagged proteins in the myristic acid–azide–enriched fraction and the corresponding whole lysates. (*C*) Gly^2 is required for S2-HA localization at the cell membrane. Shown are immunofluorescence confocal images of JTAg cells transfected to express the indicated proteins.

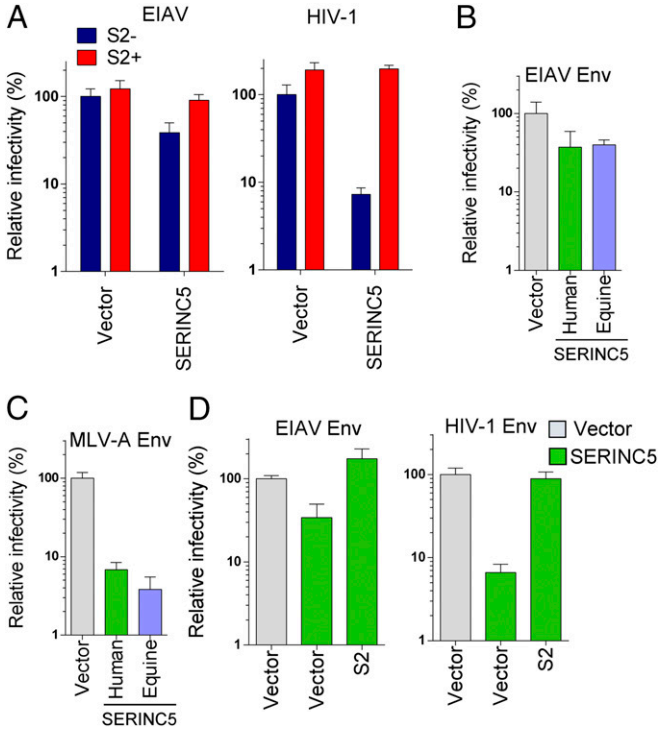

Fig. 6. EIAV env confers virus particles with partial resistance to SERINC5. (*A*) EIAV is less susceptible than HIV-1 to SERINC5 inhibition. Shown is the inhibition of the infectivity of S2-defective EIAV (*Left*) and Nef-defective HIV-1 (*Right*) virion particles by SERINC5 and the counteraction by S2. (*B* and *C*) The sensitivity of S2-defective EIAV (*B*) and S2-defective EIAV pseudotyped with MLV-A env (*C*) to human and equine SERINC5. (*D*) The susceptibility of HIV-1 particles to SERINC5 when pseudotyped with EIAV- or HIV-1 env in the absence of S2. All panels show the infectivity of single-cycle EIAV and HIV-1 virus particles produced from HEK293T cells transfected to express SERINC5 and S2 as indicated. Infectivity is expressed as the percentage relative to the infectivity of Nef-defective HIV-1_{NL4-3} or S2-defective EIAV produced in the presence or absence of SERINC5 overexpression. Error bars represent the SD of the mean calculated from quadruplicate determinations.

SERINC5-susceptible env, EIAV virions also become fully susceptible to the host factor. This result demonstrates that EIAV cores are not inherently resistant to SERINC5 and that env plays a crucial role in modulating sensitivity to SERINC5. We therefore assessed the ability of EIAV env to alter HIV-1 sensitivity to SERINC5 by studying the inhibition of HIV-1 virion particles bearing EIAV env. Although Nef-defective HIV-1 carrying an HIV-1_{HXB2} env was inhibited 15-fold by SERINC5, the same virion particles carrying EIAV env were affected only threefold by the host factor (Fig. 6*D*), mirroring the effect observed on EIAV vector particles (Fig. 6*A*). EIAV env therefore confers partial SERINC5 resistance to retrovirus particles. Confirming its anti-SERINC5 activity, expression of S2 counteracted the effect of SERINC5, irrespective of the env glycoprotein used (Fig. 6*C*).

Discussion

In this study we have established that S2 from EIAV is an infectivity factor functionally related to Nef of primate lentiviruses and to glycosylated Gag of gammaretroviruses. S2 therefore is a third example of a retroviral factor capable of promoting retrovirus infectivity by counteracting the SERINC family of proteins. Remarkably, the three retroviral factors share no sequence homology and originate from distinct and unrelated genetic regions of the retrovirus genome, indicating that the ability to counteract SERINC5 has emerged independently in primate lentiviruses, gammaretroviruses, and EIAV. The need to antagonize SERINC5 therefore has shaped the genomes of diverse retroviruses.

Despite the absence of sequence similarity, S2, Nef, and glycoGag share two crucial features: the presence of putative motifs capable of recruiting clathrin adaptors (13) and the ability to localize at the cell membrane. To counteract SERINC5, S2 requires an ExxxLL motif, which is predicted to interact with the clathrin adaptor AP2. ExxxLL is not conserved in all S2 alleles, because it is absent in the $EIAV^{LIA}$ lineage (Fig. 4*D*). However, in this case, ExxxLL is replaced by YxxL, making the ability of the canonical motif to engage with the clathrin adaptor complex a fundamental feature of S2. As already shown for Nef (17), our results confirm that these infectivity factors may function as "adaptors of adaptors" that are capable of connecting a targeted cargo [such as CD4 (18), MHC-I (19), or SERINC5 and SERINC3] to clathrin adaptors. Accordingly, it would be interesting to investigate whether S2 exerts a multifunctional role by targeting other membrane proteins, such as the EIAV receptor, or molecules crucial for the immune response, such as MHC-I. This model also implies that Nef, glycoGag, and S2 have the ability to interact with SERINC5 and SERINC3; this ability remains to be experimentally proven.

We have shown that, as can Nef, S2 can be myristoylated. As observed with Nef (4), myristoylation is essential for S2's counteraction of SERINC5. Recruitment of S2 at the plasma membrane therefore could be a prerequisite for facilitating the interaction with AP2 and recruiting SERINC5, following a model described in detail for Nef and CD4 (18). Intriguingly, a canonical myristoylation signal predicting that Ser^6 or Thr^6, in addition to Gly^2, is absent at the N terminus of S2 makes it an unusual substrate for the human *N*-myristoyl transferase, similar to other proteins such as the cAMP-dependent kinase (20) and another retroviral protein such as MLV Gag (21).

Surprisingly, irrespective of S2, EIAV infectivity is affected only minimally by SERINC5. Such increased resistance to the host factor depends on the EIAV env, reminiscent of the glycoproteins derived from some HIV-1 isolates and RNA viruses such as VSV and Ebola. EIAV env has been reported to promote virus entry via a pH-dependent pathway (22), thus reinforcing the hypothesis that the endosomal uptake could provide a port of entry that is less sensitive to SERINC5 inhibition (4). It remains to be established whether resistance to SERINC5 is a general feature shared by all EIAV isolates. Nevertheless, the question remains: Why did EIAV and HIV evolve apparently redundant mechanisms to bypass inhibition by SERINC5? Such antagonist redundancy is not unique, because HIV-1 VpU and Nef also have evolved complementary abilities to interfere with the cell-surface level of the HIV-1 receptor CD4 (23). Therefore redundancy could be necessary in some cases. Alternatively, it is possible that, in addition to its effect on retrovirus infectivity, SERINC5 exerts other activities that are similarly adverse to virus replication but are not counteracted by env. BST2, for example, was found to act both as a virus-tethering factor, which prevents the release of virion particles into the extracellular space, and as a viral sensor, which via NF-kB signals the presence of budding virus particles (24). Moreover, it would be illuminating to investigate the requirement for S2 and the effect of SERINC5 on EIAV replication in cells derived from the equine native host, accounting for the possibility that other host-specific factors could modulate SERINC5 activity against EIAV.

In conclusion, the identification of a third example of a retrovirus that independently evolved action opposing SERINC5 and SERINC3 demonstrates that these host factors have a crucial inhibitory activity directed at diverse retroviruses infecting different hosts. Taken together, these findings highlight a host–pathogen interface that should be investigated further to explore its utility in the development of innovative antiviral strategies.

Materials and Methods

Plasmids. For all experiments performed with HIV-1, NL4-3–derived plasmids carrying a frameshift mutation to disrupt the expression of *env* and/or *nef* were used as previously described (25). Env-defective viruses were complemented with a PBJ5 plasmid expressing HIV-1^{HXB2} env. The FBASA vector was used to express Amphotropic (4070-A) MLV env (26), and pMD.G was used to express VSV-G (27). The EIAV codon-optimized envelope pLGcoSUTM was constructed by inserting codon-optimized surface protein (SU) and transmembrane (TM) sequences from pSPEIAV19 (GenBank accession no. EIU01866) into the low-copy-number plasmid pLG228/30, as previously described (28). pCXCR4-FLAG has been described previously (29). An *env*-defective NL4-3 expressing S2 in place of Nef was also generated by substituting the first 73 codons of *nef* with the coding sequence from S2 derived from SPEIAV19 (GenBank accession no. U01866) (10). A codon-optimized version of S2 from SPEIAV19 and from pEV53D (30) (GenBank accession no. M87581, derived from Wyoming strain EIAV) was obtained through a seven-step PCR to substitute the required bases following human codon use and was cloned into PCDNA3.1. An HA tag also was added at the C terminus by PCR. All S2 mutants with an HA tag fused at the C terminus (L27,28AA, G2A, P26,29AA) were generated by site-directed mutagenesis. SERINC5-HA was expressed from pBJ6 (a derivative of PBJ5 that provides a low level of expression in HEK 293T cells), and pSERINC5-GFP vectors were previously described (4). mRFP-Rab7 (Addgene plasmid no. 14436) (31) was a gift from Ari Helenius, Swiss Federal Institute of Technology (ETH), Zurich. EIAV transfer vector pEIAV-SIN6.1 CGFPW (32) and the gag-pol–expressing construct pEV53D (Addgene plasmids nos. 44171 and 44168, respectively) were a gift from John Olsen, University of North Carolina, Chapel Hill, NC. pEV53D, in which the S2 ORF was disrupted, was obtained by enzymatic digestion of a unique BamHI site followed by Klenow fill-in and ligation, to insert a frameshift disrupting the S2 ORF from codon 18. Equus SERINC5 (GenBank accession no. XM_001503874) was custom synthetized (GeneArt; Life Technologies) with codons optimized for human use. The AP180 C terminus transdominant mutant was a gift from Harvey McMahon, Laboratory of Molecular Biology-Medical Research Council, Cambridge, United Kingdom (11). Dyn2K44A, Nef G2A, hCypA, and PBJ5 Nef-HA were used as described previously (25).

Cell Lines. For information about cell lines, see *SI Materials and Methods*.

Viruses and Infectivity Assay. HIV-1 limited to a single round of replication or EIAV vectors was produced by the transfection of JTAg and CEM174X cells (electroporation) or HEK293T cells (calcium phosphate transfection) as described in *SI Materials and Methods*. Viruses were quantified using the SG-PERT reverse transcription assay (33) modified as described previously (34), diluted three- or fivefold in a series of six steps, and used to infect TZM-zsGreen reporter cells Infectivity was assayed as a function of zsGreen-positive cells scored using the High Content Imaging System Operetta (Perkin-Elmer). Infectivity was calculated by dividing the number of infected cells in a well by the amount of reverse transcriptase activity associated with the virus inoculum, measured in milliunits (mU) (33). When indicated, results were expressed as a percentage of an internal control sample. For a detail description of the procedure, see *SI Materials and Methods*.

Immunofluorescence and Western Blotting. Standard procedures were used for immunofluorescence and Western blotting. For details see *SI Materials and Methods*.

Myristic Acid–Azide Pulse Labeling and Conjugation. HEK293T cells were pulsed with 50 μM myristic acid–azide (Life Technologies) for 24 h, and the incorporated azide moiety was conjugated to the alkyne immobilized on a matrix using the Click-iT protein enrichment kit (Life Technologies), as reported previously (35). HA-tagged proteins in the enriched fraction were detected by immunoblotting. For a detail description of this procedure, see *SI Materials and Methods*.

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