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Differential Receptor Usage of Small Ruminant Lentiviruses in Ovine and Caprine Cells: Host Range but not Cytopathic Phenotype Is Determined by Receptor Usage

Isidro Hötzel[1] and William P. Cheevers

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

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The ovine maedi-visna (MVV) and caprine arthritis-encephalitis (CAEV) small ruminant lentiviruses (SRLV) exhibit differential species tropism and cytopathic effects *in vitro.* Icelandic MVV-K1514 is a lytic SRLV which can infect cells from many species in addition to ruminants, whereas a lytic North American MVV strain (85/34) as well as nonlytic MVV strain S93 and CAEV can infect only ruminant cells. In the present study, we determined if differential receptor usage in sheep and goat cells is the basis of differential species tropism or cytopathic phenotype of SRLV. Infection interference assays in sheep and goat synovial membrane cells using pseudotyped CAEV vectors showed that North American MVV strains 85/34 and S93 and CAEV use a common receptor (SRLV receptor A), whereas MVV-K1514 uses a different receptor (SRLV receptor B). In addition, human 293T cells expressing CAEV but not MVV-K1514 envelope glycoproteins fused with a goat cell line persistently infected with MVV-K1514, indicating that MVV-K1514 does not use SRLV receptor A for cell-to-cell fusion. Therefore, our results indicate that the differential species tropism of SRLV is determined by receptor usage. However, receptor usage is unrelated to cytopathic phenotype.

Key Words: visna; CAEV; lentivirus; retrovirus; interference; receptors; vector; envelope.

INTRODUCTION

The ovine maedi-visna (MVV) and caprine arthritis-encephalitis (CAEV) lentiviruses cause progressive synovitis, pneumonia, mastitis, and encephalitis in sheep and goats (Crawford *et al.,* 1980; Narayan *et al.,* 1980, 1993). Like other lentiviruses, small ruminant lentiviruses (SRLV) infect cells of the monocyte/macrophage lineage and dendritic cells (Gendelman *et al.,* 1985, 1986; Gorrel *et al.,* 1992; Narayan *et al.,* 1983; Ryan *et al.,* 2000). However, MVV can also enter other cell types *in vivo,* indicating that expression of MVV receptors is not restricted to monocytes, macrophages, and dendritic cells (Brodie *et al.,* 1995; Georgsson *et al.,* 1989). Indeed, the long terminal repeat appears to be a major determinant of MVV tropism *in vitro* and may be an important factor in MVV pathogenesis (Agnarsdóttir *et al.,* 2000). Whether receptors used for entry also have a role in the pathogenesis of SRLV infections is not known.

Infection of cells *in vitro* by different SRLV strains results in either complete lysis of monolayers or persistent nonlytic infection with syncytium formation (Quérat *et al.,* 1984). CAEV strains are nonlytic while MVV strains can be either lytic or nonlytic. The basis for the differential cytopathic phenotype of SRLV is not known but could be related to receptor usage, similar to the relationship between receptor usage and cytopathic phenotype of avian retroviruses (Adkins *et al.,* 2001; Dorner and Coffin, 1986). In this regard, a previous study showed that virions from the lytic MVV-K1514 have different binding sites on sheep and goat cells than virions from the nonlytic MVV-S93 and CAEV, suggesting the possibility of a differential SRLV receptor usage related to cytopathic phenotype (Jolly and Narayan, 1989). SRLV also differ in host range *in vitro,* consistent with a differential receptor usage of SRLV (Hötzel and Cheevers, 2001). However, host range is not related to cytopathic phenotype as the lytic MVV-K1514 can infect cells from a broad range of species whereas the lytic MVV-85/34, similar to CAEV and MVV-S93, can only infect cells of ruminant species (Hötzel and Cheevers, 2001; Mselli-Lakhal *et al.,* 2000). Thus, if SRLV cytopathic phenotype is related to receptor usage, lytic MVV strains use multiple receptors which are different from the nonlytic SRLV receptor(s). Alternatively, either cytopathic phenotype or host range is not related to receptor usage.

Here we determined the receptor usage of SRLV strains of different cytopathic phenotypes and host range in sheep (SSM) and goat (GSM) synovial membrane cells. Receptor usage of CAEV and MVV strains was determined by infection interference assays using pseudotyped CAEV vectors and syncytial assays. We show that lytic and nonlytic North American MVV strains and CAEV use a common receptor for entry while the lytic MVV-K1514 uses a second receptor, indicating that

[1] To whom correspondence and reprint requests should be addressed. Telephone: (509) 335-6072. Fax: (509) 335-8529. E-mail: ihe@vetmed.wsu.edu.

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SRLV receptor usage is related to host range but not cytopathic phenotype.

RESULTS

Production of recombinant soluble CAEV and MVV envelope surface glycoproteins (SU)

We produced recombinant soluble SU from four SRLV strains for the interference assays: CAEV-63, the lytic and nonlytic North American MVV strains 85/34 and S93, and the lytic Icelandic MVV-K1514. Recombinant soluble SU was produced by transfecting 293T cells with envelope plasmids with stop codons or frameshift mutations in the transmembrane subunit (TM) just before the TM anchor (Fig. 1A). Production of recombinant soluble SU was assessed by immunoprecipitation of supernatants from transfected cells labeled with [^{35}S]methionine. Immune serum from CAEV-infected goat 8517 immunoprecipitated glycoproteins of ~135 kDa from supernatants of cells transfected with plasmids pCMV63S, pCMV93S, pCMV34S, or pCMV1514S, expressing soluble SU from CAEV-63, MVV-S93, MVV-85/34, and MVV-K1514, respectively (Fig. 1B, lanes 2, 4, 6, and 8), but not from supernatants of cells transfected with control plasmid pCR3 (Fig. 1B, lane 10). In contrast, serum from uninfected control goat 8505 did not immunoprecipitate any labeled proteins from the supernatants of transfected 293T cells (Fig. 1B, lanes 1, 3, 5, and 7). The CAEV-63, MVV-S93, and MVV-K1514 recombinant SU comigrated with the SU of wild-type CAEV-63 grown in GSM cells (Fig. 1B, lanes 2, 4, 8, and 9) while the recombinant MVV-85/34 SU migrated somewhat faster (Fig. 1B, lane 6), as previously observed for wild-type MVV-85/34 SU expressed by infected GSM cells (unpublished data). Serum 8517 also immunoprecipitated TM glycoproteins of the expected size from the supernatants of cells transfected with pCMV63S, pCMV93S, and pCMV34S, migrating as broad bands in the 25-kDa regions as well as discrete bands in the 20-kDa region (Fig. 1B, lanes 2, 4, and 6), in contrast to the native monomeric and oligomeric TM forms migrating in the 38- and 90-kDa regions, respectively (Fig. 1B, lane 9) (McGuire *et al.,* 1992). A weak band in the 36-kDa region was observed in immunoprecipitates of MVV-K1514 Env supernatants that may represent oligomerized forms of truncated TM (Fig. 1B, lane 8). The results confirm production of recombinant CAEV and MVV soluble SU and TM by transfected 293T cells.

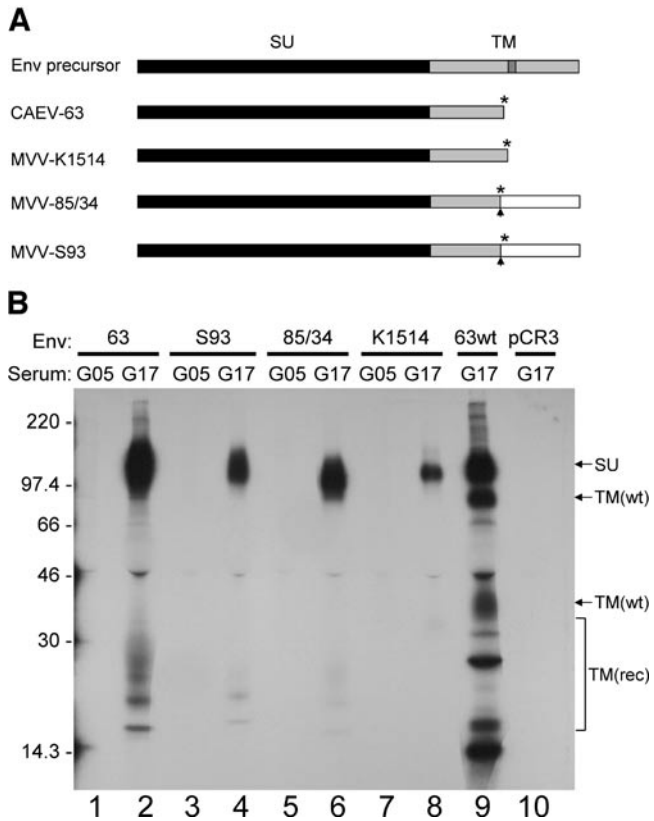

FIG. 1. Production of soluble recombinant envelope glycoproteins. (A) Schematic representation of recombinant soluble Env glycoprotein constructs. The SU and TM domains are shown in black and gray, respectively. The TM transmembrane anchor in the wild-type glycoproteins is indicated by a dark gray box. Frameshift mutations are indicated by arrowheads and stop codons by asterisks. The white boxes represent nontranslated *env* sequences downstream from introduced stop codons. (B) SDS–PAGE analysis of immunoprecipitated MVV recombinant soluble envelope glycoproteins. Human 293T cells were transfected with plasmids pCMV63S and pCRev63 (63, lanes 1 and 2), pCMV93S (S93, lanes 3 and 4), pCMV34S (85/34, lanes 5 and 6), pCMV1514S (K1514, lanes 7 and 8), or pCR3 (lane 10) and labeled with [^{35}S]methionine. Filtered supernatants of transfected cells were immunoprecipitated with serum from CAEV-63-infected goat 8517 (G17, lanes 2, 4, 6, 8, and 10) or from uninfected goat 8505 (G05, lanes 1, 3, 5, and 7). Lane 9 (63wt) shows an immunoprecipitation of ^{35}S-labeled CAEV-63 with serum 8517. The positions of SU and wild-type TM [TM(wt)] glycoproteins from CAEV-63 are indicated on the right by arrows. The positions of truncated recombinant TM glycoproteins [TM(rec)] are indicated on the right by a bracket. The positions of ^{14}C-labeled molecular markers are shown on the left.

CAEV and North American MVV strains share a common receptor in sheep and goat cells

Receptor usage of SRLV strains was determined by infection interference assays using a modification of a previously described assay (Hullinger, 1993; Hullinger *et al.,* 1993). GSM or SSM cells were incubated with soluble SU and challenged with pseudotyped virus to determine whether receptors used for entry by each strain are bound and blocked by homologous or heterologous soluble SU. For virus challenge we used vector CAEVneo pseudotyped with CAEV or MVV envelopes (Hötzel and Cheevers, 2001). The use of CAEVneo allowed the direct quantitation of virus entry events by counting the number of G418-resistant colonies after staining with crystal violet. In the interference assays, the number of available receptors, and thus virus infectivity, is reduced according to the concentration of soluble SU and its receptor-binding affinity. However, these assays are also time-

TABLE 1

Interference Assays of Pseudotyped CAEVneo with Recombinant Soluble SU in GSM and SSM Cells

Soluble SU[a]	Percentage Infectivity of Pseudotype[b]				
	CAEVneo(CO)	CAEVneo(S93)	CAEVneo(85/34)	CAEVneo(K1514)	CAEVneo(VSV)
None (pCR3)					
GSM	100.0 ± 17.0	100.0 ± 2.5	100.0 ± 3.7	100.0 ± 1.9	100.0 ± 3.1
SSM	100.0 ± 3.1	100.0 ± 3.9	100.0 ± 1.9	100.0 ± 8.4	100.0 ± 1.1
CAEV-63					
GSM	**5.2 ± 2.2**	**9.1 ± 4.1**	85.5 ± 2.5	91.8 ± 3.1	103.7 ± 2.3
SSM	**18.8 ± 3.1**	**58.2 ± 2.7**	94.6 ± 1.0	84.1 ± 5.2	96.5 ± 4.6
MVV-S93					
GSM	**8.1 ± 3.7**	**15.7 ± 0.8**	85.5 ± 5.8	93.0 ± 5.8	103.6 ± 4.1
SSM	**0 ± 0**	**14.1 ± 3.6**	**75.2 ± 0.6**	90.4 ± 5.2	91.2 ± 4.2
MVV-85/34					
GSM	**0 ± 0**	**4.1 ± 0.8**	**62.7 ± 3.7**	92.2 ± 1.9	96.3 ± 2.3
SSM	**0 ± 0**	**5.9 ± 1.2**	**51.0 ± 1.3**	89.6 ± 8.4	96.5 ± 1.1
MVV-K1514					
GSM	107.4 ± 7.6	90.9 ± 3.6	95.4 ± 4.3	108.9 ± 0.7	100.9 ± 1.4
SSM	95.3 ± 8.2	100.4 ± 1.2	99.0 ± 1.6	101.6 ± 2.0	97.9 ± 0.4

[a] Supernatants of transfected 293T cells containing recombinant soluble SU were used undiluted. The same batches of SU of each strain were used for assays in GSM and SSM cells.

[b] Values represent titers of CAEVneo in cells incubated with soluble SU as a percentage of titers in cells incubated with control (pCR3) supernatants (mean ± range; $n = 2$). Highlighted numbers indicate ≥25% titer reduction. Representative results of two experiments performed in duplicate are shown.

dependent as maximal virus entry will eventually occur regardless of the presence of soluble SU. Thus, one critical parameter for this assay is the time of exposure of cells to virus. Incubation of GSM cells with CAEVneo pseudotyped with SRLV envelopes for 1 h at 37°C usually yielded titers that were 30 to 50% of the titers obtained by incubating cells with virus for 24 h (not shown). Therefore, incubation of virus with cells for 1 h should not overcome the blocking activity of soluble SU, as previously shown for GSM cells incubated with recombinant CAEV-63 SU and CAEV-63 (Hullinger *et al.,* 1993). Another important parameter to achieve maximum inhibition in this type of assay is the time required for SU–receptor binding to achieve equilibrium. The binding kinetics of CAEV and MVV SU to their receptors are not known. However, previous results showed that addition of CAEV-63 to GSM cells simultaneously or 1 h after addition of purified CAEV-63 SU does not significantly affect the 50% inhibitory concentration (IC_{50}) of SU (Hullinger, 1993), indicating that equilibrium or near-equilibrium conditions are achieved in less than 1 h at 37°C. Therefore, for the interference assays, GSM and SSM cells were incubated with soluble SU for 1 h at 37°C, challenged with 100 colony-forming units (CFU) of pseudotyped CAEVneo (multiplicity of infection $\sim 5 \times 10^{-4}$ CFU per cell) in the presence of SU for 1 additional h at 37°C, washed, incubated in growth medium for 24 h, and then grown in selective medium.

The CAEV-CO pseudotype [CAEVneo(CO)] titers in GSM or SSM cells incubated with recombinant soluble SU of CAEV or North American MVV strains 85/34 and S93 ranged from 0 to 18.8% of the titer of CAEVneo(CO) in cells incubated with control supernatant (Table 1), indicating efficient cross-interference between these strains. As expected, recombinant soluble SU preparations did not significantly reduce the titers of CAEVneo(VSV) in GSM or SSM cells (Table 1), indicating that interference in these assays occurs at the entry level. Thus, CAEV and North American MVV strains share at least one receptor in GSM and SSM cells, tentatively designated SRLV receptor A.

Results of interference assays with CAEVneo(S93) were generally similar to those with interference assays with CAEVneo(CO), except that residual titers were somewhat higher with CAEVneo(S93) (Table 1). CAEVneo(S93) titers in cells incubated with soluble SU from CAEV or North American MVV strains ranged from 4.1 to 15.7% of the titers in cells incubated with control supernatant except for SSM cells incubated with CAEV-63 SU, in which CAEVneo(S93) titers were 58.2% compared to controls (Table 1). The less efficient interference of CAEVneo(S93) by CAEV-63 SU in SSM cells could be due to the presence of an additional receptor for MVV-S93 in SSM cells not bound by CAEV-63 SU or a lower affinity of CAEV-63 SU in SSM compared to GSM cells. To determine whether incubation of SSM cells with higher CAEV-63 SU concentrations would further reduce MVV-S93 infectivity, we performed interference assays with serial dilutions of purified native CAEV-63 SU in SSM cells. Similar to the results obtained with undiluted re-

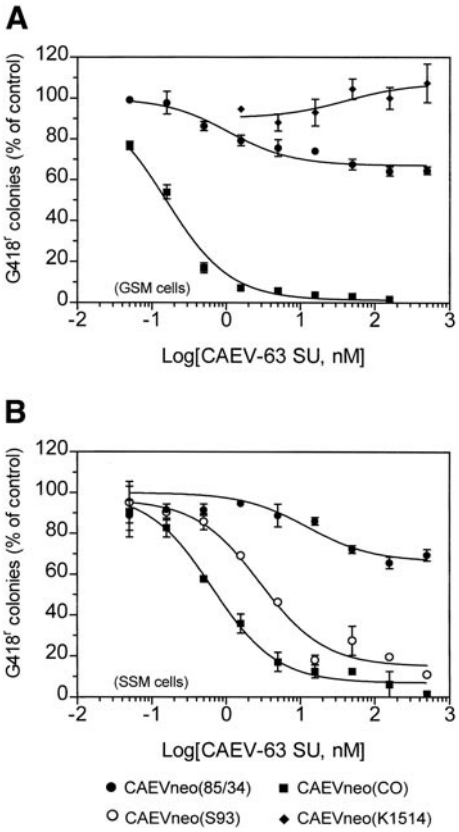

FIG. 2. Interference assays using serial dilutions of soluble SU in GSM (A) and SSM (B) cells. Cells were incubated with serial dilutions of purified CAEV-63 SU in DMEM with 10% FBS for 1 h, challenged with 100 CFU of pseudotyped CAEVneo in the presence of SU for 1 additional h, washed twice in DMEM, and processed for titration as previously described. Each point represents the average ($n = 2$) and range of titers of each virus in cells incubated with soluble SU relative to the titer of each virus in cells incubated with 60 μg/ml of BSA in DMEM. Data were fitted by nonlinear regression using a one-site competition model with GraphPad Prism software, Version 2.01.

combinant soluble CAEV-63 SU shown in Table 1, incubation of SSM cells with 5 nM purified native CAEV-63 SU was sufficient to decrease the titers of CAEVneo(CO) by ~83%, while CAEVneo(S93) titers were reduced by only 53% (Fig. 2B). However, higher concentrations of CAEV-63 SU reduced infectivity of CAEVneo(S93) in SSM cells by up to 89% (Fig. 2B), indicating that MVV-S93 does not efficiently use a receptor in SSM cells that is not bound by CAEV-63 SU. Thus, CAEV and MVV-S93 share the same set of receptors in GSM and SSM cells.

Unlike the efficient interference observed for CAEVneo(CO) and CAEVneo(S93) by their respective homologous recombinant soluble SU and by MVV-85/34 SU, titers of CAEVneo(85/34) were only partially reduced by MVV-85/34 SU in either GSM or SSM cells (Table 1). In addition, MVV-S93 SU partially reduced CAEVneo(85/34) titers only in SSM cells, while recombinant soluble CAEV-63 SU partially reduced CAEVneo(85/34) titers only in GSM cells (Table 1). To determine whether this partial or absent interference of CAEVneo(85/34) was due to low SU concentrations or a one-way interference between these strains, interference assays of CAEVneo(85/34) were performed using serial dilutions of purified native CAEV-63 SU. The ability of MVV-85/34 SU to efficiently block CAEVneo(CO) infectivity in GSM and SSM cells indicated that MVV-85/34, like CAEV and MVV-S93, also uses SRLV receptor A for infection. Thus, some interference of CAEVneo(85/34) by CAEV-63 would be expected. In fact, a partial reduction of CAEVneo(85/34) titers by purified native CAEV-63 SU was observed in GSM and SSM cells (Figs. 2A and 2B). This partial reduction of CAEVneo(85/34) titers was observed even at CAEV-63 SU concentrations as high as 500 nM, while CAEVneo(CO) was almost completely blocked by CAEV-63 SU at 16 nM (Figs. 2A and 2B). Increasing concentrations of CAEV-63 SU over 50 nM did not appear to further reduce titers of CAEVneo(85/34). The 95% confidence intervals for the maximal inhibition of CAEVneo(85/34) by CAEV-63 SU in GSM and SSM cells in the experiments shown in Fig. 2 were 33 $\pm$ 3.3% and 33.5 $\pm$ 8.3%, respectively.

Binding of MVV-85/34 to sheep and goat cells does not depend on receptors used for entry

Although MVV-85/34 uses SRLV receptor A for entry, the results in Figs. 2A and 2B suggest that MVV-85/34 Env can bind a second receptor in GSM and SSM cells. One interpretation is that MVV-85/34 uses a second receptor for entry. An alternative possibility is that MVV-85/34, but not other SRLV strains, remains bound to GSM or SSM cells during challenge in the presence of SU and then infects the cells upon withdrawal of soluble SU. To test this second possibility, GSM cells were incubated with serial dilutions of CAEV-63 SU for 1 h and then challenged for 1 h with CAEVneo(85/34). Unbound virus and SU were then aspirated and cells washed and incubated for 24 h in growth medium with or without 160 nM purified CAEV-63 SU before G418 selection. Similar to the results in Fig. 2A, incubation of GSM cells with CAEV-63 SU only during the period of challenge decreased the infectivity of CAEVneo(85/34) by a maximum of only 54% (Fig. 3A). However, CAEV-63 SU added after unbound virus was washed from the cells inhibited CAEVneo(85/34) entry completely (Fig. 3A). Similar results were also obtained with SSM cells (not shown). Thus, the MVV-85/34 envelope can mediate binding but not infection of GSM and SSM cells independently of SRLV receptor A, allowing infection of cells through SRLV receptor A after soluble SU is removed.

MVV-K1514 is resistant to interference by CAEV and North American MVV SU

Unlike CAEVneo pseudotyped with CAEV or North American MVV envelopes, CAEVneo(K1514) titers were not significantly reduced by preincubation of GSM and

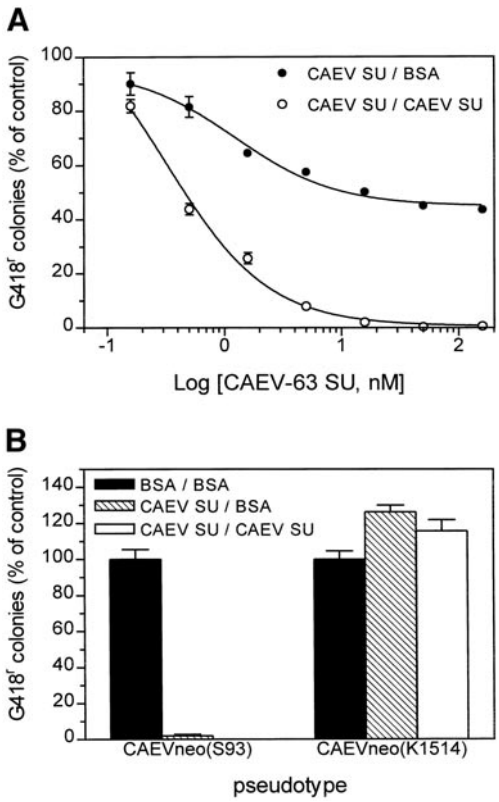

FIG. 3. Interference assays in the continued presence of soluble CAEV-63 SU. (A) GSM cells were incubated with serial dilutions of CAEV-63 SU in DMEM–10% FBS for 1 h (shown in x-axis), challenged with 100 CFU of pseudotyped CAEVneo(85/34) in the presence of CAEV-63 SU for 1 additional h, washed twice with DMEM, and incubated in DMEM–10% FBS with 160 nM BSA (CAEV SU/BSA, solid circles) or 160 nM CAEV-63 SU (CAEV SU/CAEV SU, open circles) before G418 selection. Points represent average ($n = 2$) of the titer of virus in cells incubated with different CAEV-63 SU concentrations relative to titer in cells incubated without SU during challenge. (B) GSM cells were incubated with 500 nM CAEV-63 SU or BSA for 1 h, challenged with 100 CFU of pseudotyped CAEVneo(S93) or (K1514) in the presence of CAEV-63 SU for 1 additional h, washed twice with DMEM, and incubated in DMEM–10% FBS with 160 nM BSA (CAEV SU/BSA, cross-hatched bars) or CAEV-63 SU (CAEV SU/CAEV SU, white bars) before G418 selection. Titers are normalized with the titer of pseudotyped virus in GSM cells preincubated with BSA (BSA/BSA, black bars). Error bars in A and B and represent titer ranges.

SSM cells with CAEV-63, MVV-S93, or MVV-85/34 recombinant soluble SU (Table 1). To determine whether higher concentrations of SU can block CAEVneo(K1514) infection, interference assays of this pseudotype were also performed with serial dilutions of purified CAEV-63 SU. As shown in Fig. 2A, higher concentrations of CAEV-63 SU were not able to decrease the titers of CAEVneo(K1514) in GSM cells, suggesting that MVV-K1514 either does not use SRLV receptor A or uses a second receptor efficiently in addition to SRLV receptor A. As the partial inhibition of CAEVneo(85/34) by CAEV-63 SU is due to binding of virus to cells through mechanisms independent of receptors used for entry, we asked whether the failure of soluble SU to interfere with CAEVneo(K1514) was due to a similar binding and infection after removal of SU. To determine this, GSM cells were incubated with 500 nM of purified CAEV-63 SU, challenged with CAEVneo(K1514), washed, and further incubated in growth medium with or without 160 nM of purified CAEV-63 SU. Similar to the results in Fig. 2, incubation of GSM cells with CAEV-63 SU before and during the challenge period inhibited the infectivity of CAEVneo(S93) by 98% while infectivity of CAEVneo(K1514) was unaffected by preincubation with CAEV-63 SU (Fig. 3B). However, continued incubation of GSM cells with 160 nM CAEV-63 SU after removal of unbound virus completely blocked CAEVneo(S93) infectivity but did not significantly affect CAEVneo(K1514) titers (Fig. 3B). This contrasts the almost complete reduction in titer of CAEVneo(85/34) under the same conditions (Fig. 3A). The results indicate that resistance of CAEVneo(K1514) to interference by CAEV-63 SU is not due to receptor-independent binding. Instead, it appears that MVV-K1514 uses a second receptor, SRLV receptor B, for entry into sheep and goat cells.

MVV-K1514 cannot use SRLV receptor A for cell-to-cell fusion

Incubation of GSM or SSM cells with MVV-K1514 recombinant soluble SU preparations similar to the soluble SU from North American MVV strains failed to interfere with the infection of any CAEVneo pseudotype, including the homologous CAEVneo(K1514) (Table 1). Thus, a different approach was devised to test interference of SRLV by MVV-K1514 SU. While it has not been possible to derive cell lines where most or all cells are persistently infected with CAEV or North American MVV strains without cytopathic effects, at least one goat cell line has been persistently infected with the Icelandic MVV-KV1772 without cytopathic effects (Andresson *et al.,* 1993). This suggests that some cell lines can be persistently infected by Icelandic MVV strains which display homologous receptor interference. Thus, we developed a goat synovial membrane cell line persistently infected with MVV-K1514. The SV40-immortalized goat synovial membrane cell line TIGEF (Da Silva Teixeira *et al.,* 1997) was infected with MVV-K1514 at a high multiplicity of infection and the surviving cells, designated T1514 cells, were passaged uncloned. The persistently infected T1514 cell line produced infectious cytopathic MVV-K1514 and had a normal fibroblast-like morphology, although growing more slowly than the parental TIGEF cell line (not shown). As TIGEF and T1514 cells fail to form visible colonies after infection with CAEVneo(VSV), probably due to downregulation of the *neo* SV40 promoter by the SV40 large T antigen and the lower plating efficiency of the infected cells, we used cell-to-cell fusion assays to determine receptor interference. Human 293T cells were transfected with plasmids expressing MVV-K1514 or

CAEV-CO Env or control plasmid pCR3 and cocultured overnight with TIGEF or T1514 cells. TIGEF and T1514 cells cocultured with 293T cells transfected with plasmid pCR3 did not form syncytia, except for small scattered syncytia present in both normal TIGEF and T1514 cells (Figs. 4A and 4B). This indicates that cell surface expression of MVV-K1514 Env in the persistently infected T1514 cells is insufficient to induce significant fusion with the human 293T cells. As expected, TIGEF cells cocultured with 293T cells expressing MVV-K1514 Env formed large syncytia (Fig. 4C). In contrast, T1514 cells cocultured with the same 293T cells expressing MVV-K1514 did not form any significant syncytia (Fig. 4D), indicating that T1514 cells lack receptors for induction of cell-to-cell fusion by MVV-K1514. To determine whether functional SRLV receptor A is expressed on the surface of T1514 cells, we performed fusion assays with CAEV-CO Env, which recognizes this receptor. In contrast to the striking inhibition of syncytia formation by MVV-K1514 Env in T1514 cells, CAEV-CO Env was able to induce syncytia of the same size and to the same extent in either TIGEF or T1514 cells (Figs. 4E and 4F), indicating that T1514 cells do express functional SRLV receptor A. Collectively, the results indicate that MVV-K1514 cannot use SRLV receptor A for cell-to-cell fusion.

DISCUSSION

Infection and syncytium interference assays have been used to determine common and differential receptor usage of retroviruses when the identity of the receptors is not known (Miller and Walgamot, 1997; Sommerfelt and Weiss, 1990). These assays are usually performed using infected cell lines with viral receptors blocked by endogenously produced Env, which display interference with homologous superinfection and formation of syncytia. However, most SRLV strains cannot establish persistently infected cell lines without continuous cell-to-cell fusion or lysis. In addition, cells acutely infected with MVV-K1514 are permissive to homologous superinfection (Haase *et al.,* 1982), a characteristic shared with other cytopathic retroviruses (Donahue *et al.,* 1991; Pauza *et al.,* 1990; Weller *et al.,* 1980). These problems were solved by using exogenous soluble SU to block host cell receptors. Soluble SU from CAEV and North American MVV strains could efficiently block homologous receptors in infection interference assays. However, the undiluted soluble SU of MVV-K1514 did not block the receptors used by any SRLV pseudotype, including the homologous CAEVneo(K1514), in contrast, for example, with the 95% reduction of CAEVneo(CO) titers in SSM cells by MVV-85/34 soluble SU when diluted 1:100 (not shown). The failure of MVV-K1514 SU to block receptors could be due to an abnormal processing of MVV-K1514 SU or an excess of receptors for this strain in GSM and SSM cells. Alternatively, the binding affinity of monomeric MVV-K1514 SU for its receptor(s) may be low, similar to the low affinity (about 500 nM) of the human immunodeficiency virus type 1 (HIV-1) SU for CXCR4. The binding affinity of HIV-1 SU for CXCR4 is 50- to 100-fold lower than the affinity of HIV-1 SU for CD4 or CCR5, too low to detect SU-CXCR4 interactions in binding assays (Hoffman *et al.,* 2000). To overcome this problem, we exploited the ability of Icelandic MVV strains to persistently infect goat cell lines without cytopathic effects (Andresson *et al.,* 1993) by using a TIGEF cell line persistently infected with MVV-K1514 and resistant to MVV-K1514-induced fusion.

In summary, we show that CAEV and North American MVV strains 85/34 and S93 use a common receptor (SRLV receptor A) in sheep and goat cells as indicated by the efficient cross-interference observed between these strains. In contrast, the Icelandic MVV-K1514 uses a second receptor (SRLV receptor B) as indicated by failure of the SU from CAEV and North American MVV strains to block the infectivity of CAEVneo(K1514) and the ability of CAEV but not MVV-K1514 Env to induce syncytia in cells persistently infected with MVV-K1514. Consistent with the interpretation that SRLV use at least two different receptors to infect GSM and SSM cells, we have recently found that hamster × sheep somatic cell hybrid clones retaining sheep chromosomes conferring susceptibility to CAEVneo(K1514) are completely resistant to CAEVneo(85/34) (Hötzel and Cheevers, 2002).

Our results confirm and extend the results of a study describing different binding sites for whole virions from the nonlytic CAEV and MVV-S93 and the lytic MVV-K1514 in sheep and goat cells (Jolly and Narayan, 1989). We show that differential SRLV binding sites described in that study reflect at least two different bona fide receptors used for entry and syncytium formation in sheep and goat cells. However, unlike other retroviruses, SRLV receptor usage is not related to cytopathic phenotype, as the lytic MVV-85/34 and the nonlytic CAEV-CO and MVV-S93 used SRLV receptor A for entry while the lytic MVV-K1514 used SRLV receptor B. Instead, the differential SRLV receptor usage is related to the infectivity of strains to cells of different species (Hötzel and Cheevers, 2001). Whether other broadly tropic MVV strains like the British MVV-EV1 strain (Lyall *et al.,* 2000) also use SRLV receptor B remains to be determined.

A relevant question is whether the receptor usage of strains used in this study reflects the receptors used for infection of macrophages and dendritic cells *in vivo.* Determination of interference groups in cultured macrophages was not possible as drug selection requires continuous cell proliferation. However, it is likely that these receptors are used for infection of macrophages since receptors were expressed in GSM and SSM cells under normal culture conditions and not by overexpression of recombinant forms. In addition, cell types other than those of the monocyte/macrophage lineage appear

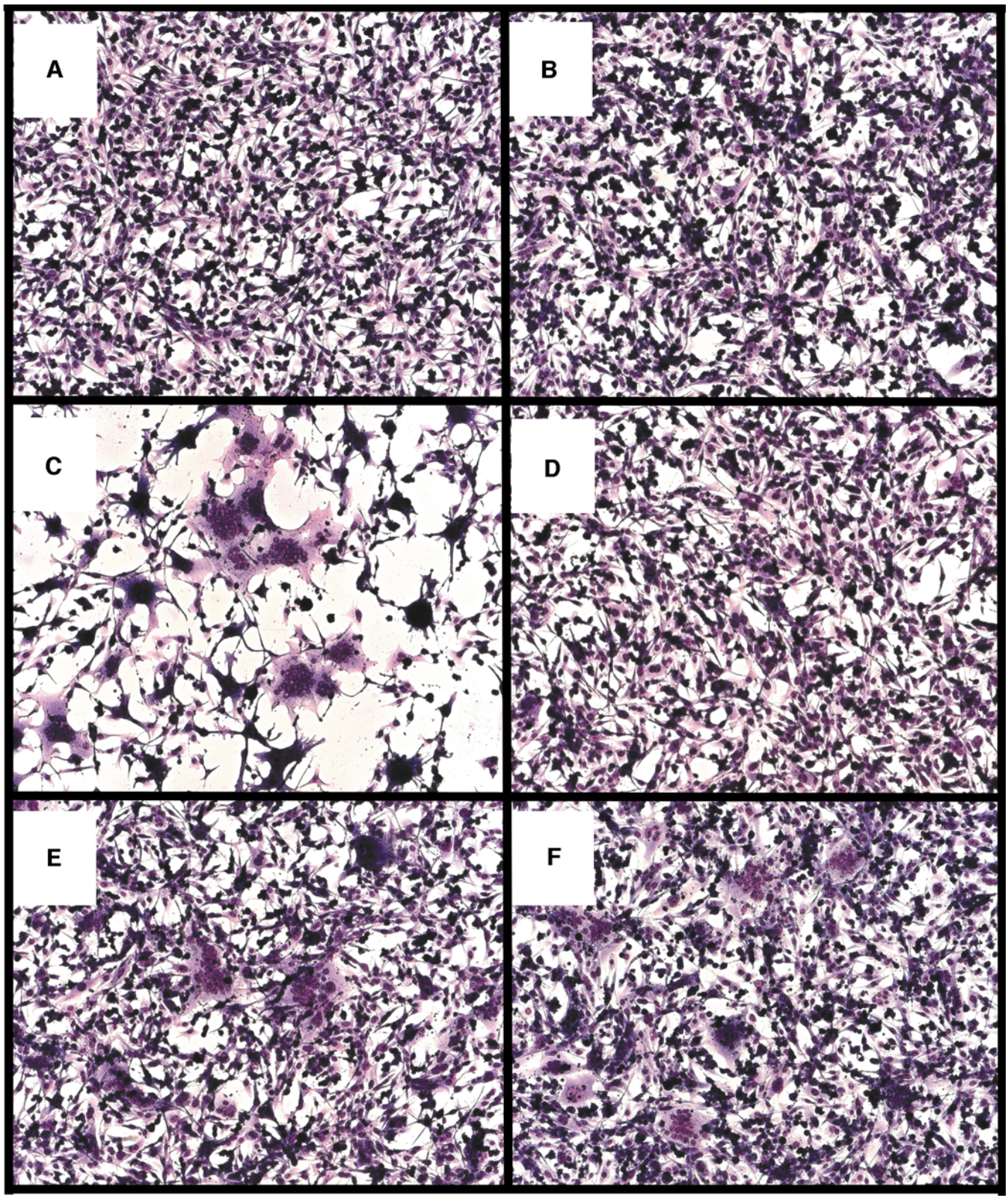

FIG. 4. Syncytial assays of MVV-K1514 and CAEV-CO Env in TIGEF or T1514 cells. TIGEF (A, C, and E) or T1514 cells (B, D, and F) were cocultured 2:1 with 293T cells transfected with control plasmid pCR3 (A and B) or plasmids expressing MVV-K1514 (C and D) or CAEV-CO Env (E and F). Cocultured cells were incubated overnight, fixed in methanol, and stained with Giemsa.

to express MVV receptors *in vivo* (Brodie *et al.,* 1995; Georgsson *et al.,* 1989).

The ability of MVV-85/34 to efficiently bind to sheep and goat cells independently of SRLV receptor A (Figs. 2 and 3A) suggests the presence of an additional receptor involved in binding, but not entry, of a subset of SRLV strains. MVV-S93 and CAEV-CO appear to lack this SRLV receptor A-independent binding, indicating that it is Env-dependent. Whether MVV-K1514 has a similar binding activity could not be determined due to the resistance of this strain to infection interference with soluble SU. This binding activity is not an artifact of pseudotyping in human cells as similar results can be observed in interference assays with wild-type MVV-85/34 and CAEV-CO grown in GSM cells using syncytium formation to score infectivity (not shown). The results in Fig. 3A indicate that the SRLV receptor A-independent binding is not sufficient for infection: CAEV-63 SU, which does not block this binding, can completely block CAEVneo(85/34) entry if added to the medium at high concentrations before and after the challenge period. We suggest that either MVV-85/34 Env itself or a host cell protein incorporated in the envelope specifically selected by MVV-85/34 Env binds a second putative receptor which mediates low-affinity binding only rather than infection. Alternatively, the SRLV receptor A-independent binding of MVV-85/34 to GSM and SSM cells could be receptor independent. In either case, the SRLV receptor A-independent binding of MVV-85/34 could be a contributing factor in the much higher infectivity of CAEVneo(85/34) relative to pseudotypes of other SRLV strains to GSM cells (Hötzel and Cheevers, 2001). Similar cell binding and enhancing activities have been described for the SU of primate lentiviruses. For instance, HIV-1 SU binds cell surface heparan sulfate proteoglycans and SU of both human and simian immunodeficiency viruses bind to the DC-SIGN and DC-SIGNR lectins (Mondor *et al.,* 1988; Geijtenbeek *et al.,* 2000; Pöhlmann *et al.,* 2001). All these binding activities can facilitate virus infection through CD4 and coreceptors. However, mannan, which binds to DC-SIGN and DC-SIGNR, did not block the residual binding of CAEVneo(85/34) to GSM cells incubated with CAEV-63 SU (not shown), indicating that these lectins are not involved in binding of MVV-85/34 to GSM cells. Interestingly, one of the proteins bound by MVV-K1514 on the surface of sheep cells is a 30-kDa proteoglycan with chondroitin sulfate side chains (Bruett *et al.,* 2000), suggesting a possible class of molecules involved in MVV-85/34 binding. Regardless of the mechanism for binding, our results showing binding of MVV-85/34 to sheep and goat cells independent of the receptor used for entry illustrate a caveat of defining SRLV receptors and interference groups based only on whole virus binding assays.

Interestingly, the interference assays suggest that MVV and CAEV SU bind with higher affinity to sheep and goat receptors, respectively. Although the IC_{50} of SU is time-dependent in this assay, two patterns were consistent in different experiments: the inhibition curves of CAEV-63 SU were shifted toward a lower IC_{50} in GSM cells relative to SSM cells by 3- to 10-fold (Figs. 2A and 2B), while the opposite was observed for the SU of both North American MVV strains, whose inhibition curves were shifted toward a higher IC_{50} in GSM cells relative to SSM cells by a similar amount (not shown, but compare the inhibition efficiencies in SSM and GSM cells in Table 1). This is reflected by the different efficiencies with which pseudotypes infect sheep and goat cells: while titers of CAEVneo(S93) and CAEVneo(85/34) were the same or somewhat higher in SSM compared to GSM cells, the titers of CAEVneo(CO) were consistently 3- to 10-fold lower in SSM cells compared to GSM cells in all titration experiments (not shown). Thus, it appears that CAEV and MVV Env, although able to mediate infection of both sheep and goat cells, bind with higher affinity to receptors from the species from which these viruses are naturally isolated. This in turn could determine the relative efficiency with which these viruses replicate in infected sheep and goats.

MATERIALS AND METHODS

Cells and virus

GSM cells were derived as previously described (Crawford *et al.,* 1980). SSM cells were derived from explants of synovial membranes from the carpal joints of a 4-month-old lamb. This lamb was seronegative for CAEV SU as determined by competitive enzyme-linked immunosorbent assay (Özyörük *et al.,* 2001). In addition, SSM cultures did not develop cytopathic effects characteristic of SRLV infection upon passaging or cocultivation with GSM cells. The human 293T cell line was obtained from Richard Sutton (Baylor College of Medicine, Houston). The TIGEF cell line was obtained from Phillipe Lena (Ecole Nationale Vétérinaire de Lyon, France). All cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS). MVV-K1514 was grown in GSM cells in DMEM with 2% FBS.

Derivation of T1514 cells

T1514 cells are TIGEF cells persistently infected with MVV-K1514 that grow with normal fibroblast-like morphology. Derivation of T1514 cells was similar to a previously described G-37 goat cell line persistently infected with MVV-KV1772 (Andresson *et al.,* 1993). Briefly, 5×10^5 TIGEF cells were seeded in a 25-cm^2 flask and infected with MVV-K1514 at a multiplicity of infection (MOI) of two 50% tissue culture infective doses ($TCID_{50}$) per cell. After the initial massive cell fusion and death, discrete clones of cells with fibroblast-like morphology were defined by resistance to MVV-K1514-induced lysis and fusion upon

infection with virus at a MOI of two $TCID_{50}$ per cell. These cells, designated T1514, were passaged uncloned in DMEM–10% FBS.

Production and titration of pseudotyped viruses

CAEVneo pseudotyped with the envelope glycoproteins of CAEV-CO, MVV-S93, MVV-85/34, MVV-K1514 or vesicular stomatitis virus were produced by cotransfection of pCAEVneo10 and envelope plasmids pCMVCO2, PCMV93, pCMV34, pCMV1514, or pMEVSVg in 293T cells as previously described (Hötzel and Cheevers, 2001). CAEVneo pseudotypes for interference assays were frozen in aliquots at −80°C and titrated on GSM and SSM cells as previously described (Hötzel and Cheevers, 2001) except that cells were incubated with virus for only 1 h and were then washed with 1 ml of DMEM without serum to remove unbound virus.

Construction of soluble Env expression plasmids

Clones encoding soluble envelope glycoproteins of North American MVV strains were constructed by introducing missense mutations in the region of *env* encoding the carboxy terminus of the TM glycoprotein ectodomain. Plasmid pCMV93S encoding a soluble MVV-S93 Env was obtained by deleting the *Hin*dIII–*Eco*RI fragment in the polylinker of pCMV93 (Hötzel and Cheevers, 2001) and introducing a +1-bp frameshift mutation in the region of *env* encoding TM by digestion of this plasmid with *Bam*HI followed by a fill-in reaction and religation. Plasmid pCMV34S encoding a soluble MVV-34 Env was also obtained by introducing a +1-bp frameshift mutation in the region of *env* encoding TM by digestion of pCMV34 (Hötzel and Cheevers, 2001) with *Nhe*I followed by a fill-in reaction and religation. A soluble CAEV Env clone was produced by PCR amplification of the CAEV-63 *env* regions encoding the SU and TM ectodomains with primers ENVF (5′-GGGAATTCGCGGCCGCGAGGAAAACTGGGTTGAAGTAACC-3′) and ENVRTM (5′-GGAGATCTTCAGAATTCAGACCTCTTTTAAGCTTTCCCATAC-3′) using plasmid pC63-14B (Knowles *et al.,* 1991) as template for 30 cycles with an annealing temperature of 50°C. The amplification product was digested with *Not*I and *Bgl*II and cloned between the same sites of expression vector VR1012 (Vical) to yield plasmid pCMV63S. This clone requires Rev in *trans* for expression of Env. The CAEV-63 *rev* gene was cloned by RT-PCR amplification of oligo$(dT)_{15}$-primed total RNA from CAEV-63-infected GSM cells, purified with the Trizol Reagent (GIBCO-BRL, Grand Island, NY). The *rev* open reading frame was amplified with primers ENVF and REVR (5′-GGGAGATCTTCATAGTGACTTGCTTTTCTTATAACACCA-3′), and the amplification product was cloned in the TA vector pCR3.1 (Invitrogen) to produce plasmid pCRev63. A soluble MVV-K1514 Env clone was produced by replacing the TM region of *env* with a PCR amplification product obtained with primers VIS3F (5′-AATATAACAGTAGGAAATGGAAC-3′) and VISTMR (5′-GGGCTCGAGTTATTTGAGCCACGAGAACCAAGAGG-3′) using plasmid pCMV1514 as template for 30 cycles with an annealing temperature of 50°C. The amplification product was digested with *Xba*I and *Xho*I and cloned between the same sites of pCMV1514 yielding pCMV1514S, truncated just before the anchor domain of TM. This clone also requires Rev in *trans* for expression of Env. The MVV-K1514 *rev* gene was cloned by RT-PCR amplification of oligo$(dT)_{15}$-primed total RNA from MVV-K1514-infected GSM cells. The *rev* open reading frame was amplified with primers VISNAENVFE and VISNAENVRX (Hötzel and Cheevers, 2001) and the amplification product was cloned between the *Eco*RI and *Xho*I sites of vector pCR3 (Invitrogen) to produce plasmid pCRevV. Plasmids were sequenced to confirm the frameshift mutations and lack of PCR misincorporations.

Production of soluble SU

Native CAEV-63 soluble SU was purified by immunoaffinity chromatography with monoclonal antibody F7-299 as previously described (Kemp *et al.,* 2000). CAEV-63 SU was more than 95% pure as determined by Coomassie blue staining following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The concentration of purified CAEV-63 SU was determined with a BCA assay kit (Pierce Chemical Co., Rockford, IL). Supernatants containing recombinant soluble Env or control supernatants were produced by transfecting 3×10^6 human 293T cells in 100-mm plates with 10 μg of pCMV63S and pCRev63, 10 μg of pCMV1514S and pCRevV, or 20 μg of pCMV93S, pCMV34S, or pCR3 by the calcium phosphate coprecipitation method. Culture medium was replaced with 16 ml of DMEM–10% FBS 18 h posttransfection, and the conditioned culture medium was harvested 72 h posttransfection, clarified by centrifugation at 5000 *g* for 15 min and stored at −80°C. For immunoprecipitation of recombinant soluble envelope glycoproteins, transfected 293T cells in 25-cm^2 flasks were incubated in 4 ml methionine-free DMEM (GIBCO) supplemented with 10% FBS and 100 μCi/ml [^{35}S]methionine. Supernatants were harvested 72 h posttransfection, clarified by centrifugation at 3000 *g* at 4°C for 20 min and filtered through a 0.2-μm filter. Immune serum from CAEV-63-infected goat 8517 and normal serum from goat 8505 (10 μl each) were used to immunoprecipitate recombinant envelope glycoproteins in supernatants (100 μl each). Immunoprecipitated proteins were resolved by SDS–PAGE in a 4–20% gradient gel under reducing conditions and detected by autoradiography.

Interference assays

GSM or SSM cells in 6-well plates (2×10^5 cells/well) were incubated with 1 ml of recombinant soluble SU or $10^{-0.5}$ serial dilutions of purified CAEV-63 SU in DMEM–

10% FBS in duplicate for 1 h at 37°C followed by the addition of 100 μl of DMEM–10% FBS containing 100 CFU of pseudotyped CAEVneo. After incubation of cells with virus and SU for 1 additional h at 37°C, cells were washed twice with 1 ml of DMEM and incubated for 24 h in DMEM–10% FBS. In some experiments, DMEM–10% FBS containing 160 nM CAEV-63 SU was added to the cells after the wash. The next day cells were detached from plates with trypsin and transferred to 60-mm plates in DMEM–10% FBS with 750 μg/ml of active G418 (GIBCO BRL). After 9 to 10 days of growth under selective conditions, cells were stained with crystal violet and colonies were counted. Incubation of GSM or SSM cells with undiluted conditioned culture medium of 293T cells transfected with control plasmid pCR3 did not significantly affect titers of any CAEVneo pseudotype compared to cells preincubated in fresh medium (not shown).

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