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The Envelope Glycoprotein of Human Endogenous Retrovirus Type W Uses a Divergent Family of Amino Acid Transporters/Cell Surface Receptors

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The human endogenous retrovirus type W (HERV-W) family includes proviruses with intact protein-coding regions that appear to be under selection pressure, suggesting that some HERV-W proviruses may remain active in higher primates. The envelope glycoprotein (Env) encoded by HERV-W is highly fusogenic, is naturally expressed in human placental syncytiotrophoblasts, and has been reported to function as a super-antigen in lymphocyte cultures. Recent evidence suggested that HERV-W Env can mediate syncytium formation by interacting with the human sodium-dependent neutral amino acid transporter type 2 (hASCT2; gene name, SLC1A5) (J.-L. Blond, D. Lavillette, V. Cheynet, O. Bouton, G. Oriol, S. Chapel-Fernandez, B. Mandrand, F. Mallet, and F.-L. Cosset, J. Virol. 74:3321-3329, 2000) and that it can pseudotype human immunodeficiency virus cores (D. S. An, Y. Xie, and I. S. Y. Chen, J. Virol. 75:3488-3489, 2001). By using cell-cell fusion and pseudotype virion infection assays, we found that HERV-W Env efficiently uses both hASCT2 and the related transporter hASCT1 (gene name, SLC1A4) as receptors. In addition, although HERV-W Env mediates only slight syncytium formation or infection of mouse cells, it utilizes the mouse transporters mASCT1 and mASCT2 when their sites for N-linked glycosylation are eliminated by mutagenesis. Consistent with their role as a battlefield in host-virus coevolution, the viral recognition regions in ASCT1 and ASCT2 of humans and mice are highly divergent compared with other regions of these proteins, and their ratios of nonsynonymous to synonymous nucleotide sequence changes are extremely large. The recognition of ASCT1 and ASCT2 despite this divergence of their sequences strongly suggests that the use of both receptors has been highly advantageous for survival and evolution of the HERV-W family of retroviruses.

Human endogenous retroviral sequences (HERVs), which have homology to known animal retroviruses, comprise a significant proportion (ca. 8%) of the human genome (8, 35, 41). These sequences probably originated from multiple primary infections of germ line cells by ancient endemic retroviruses, followed by periods of expansion within the host (13). The majority of HERVs are truncated or mutated and have thereby lost ability to produce fully functional proteins or replication-competent viruses. However, some HERVs contain long open reading frames capable of encoding complete viral proteins that even assemble into retrovirus-like particles (14, 57). Expression of HERV RNAs or proteins has been associated with diseases or inflammatory conditions, including multiple sclerosis, diabetes, autoimmune arthritis, and schizophrenia (19, 26, 31, 39, 42). In addition, several HERV proteins, particularly the envelope glycoproteins (Env), are expressed in a tissue-specific manner in normal cells (13). For example, the Env glycoproteins of the HERV type E (HERV-E), HERV-R, and HERV-W families are expressed in the syncytiotrophoblast layer of the placenta (11, 12, 30, 34).

Fully endogenized retroviruses are believed to be selectively neutral or even advantageous to their host species (13). Examples of advantageous endogenous retroviral sequences are the Fv-1, Fv-4, and Rmcf genes of mice, which confer resistance to infections by exogenous retroviruses (21, 52). However, other endogenous retroviruses can participate in infectious processes that cause disease (13), for example, in leukemogenesis of AKR mice (18), in breast cancers caused by mouse mammary tumor viruses (24), and in immunodeficiencies caused by FELIX in domestic cats (3). In general, inherited proviruses within a single family occur in a spectrum of forms, with some being fully endogenized or repressed and others being expressed in certain conditions or genetic backgrounds and potentially contributing to retrotransposition processes or to diseases.

The recently identified HERV-W family is believed to have invaded the human lineage and other Old World monkeys during the last approximately 25 to 40 million years (28, 60). It is presently represented by approximately 654 family members in the sequenced human genome (41). Although most of these are incapable of protein expression and are in many cases solitary long terminal repeats or processed pseudogenes, other HERV-W sequences have open reading frames, and at least nine have been reported to contain long *env* gene sequences (60). One of these encodes a highly fusogenic Env glycoprotein that is selectively expressed in normal syncytiotrophoblasts in

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the placenta (12, 34, 38). Based on its specific expression in this layer of the placenta composed of fused cells, it has been proposed that the HERV-W envelope may play a physiological role in placenta formation (12, 38). Evidence derived from the expressed sequence tag database also suggests that HERV-W RNAs are expressed in other tissues (11, 41). Ratios of non-synonymous to synonymous substitutions in HERV-W open reading frames have also implied that the encoded proteins are expressed and that they are subject to strong selective pressures (11, 29). Expression of HERV-W sequences has specifically been associated with multiple sclerosis and schizophrenia (26, 42). Recent evidence also suggests that the full-length HERV-W Env glycoprotein can pseudotype human immunodeficiency virus type 1 (HIV-1) virion cores (1) and that one Env variant may function as a superantigen to activate subsets of T lymphocytes (43). The HERV-W Env transmembrane subunit (TM) also contains an immunosuppressive domain (8).

Phylogenetic analysis of the HERV-W Env glycoprotein suggested that it is weakly related to a large, widely dispersed interference group of retroviruses that includes the RD114 feline endogenous virus, baboon endogenous virus (BaEV), type D simian retroviruses, and avian reticuloendotheliosis viruses, which all use the human sodium-dependent neutral amino acid transporter type 2 (hASCT2) as their common cell surface receptor (47, 56). Based on this relationship, Blond et al. (12) recently showed that cells containing HERV-W Env fuse with rodent cells that express hASCT2. Although HERV-W Env can also pseudotype lentiviral vectors to form infectious virions able to transduce CD4-negative human cells (1), it has not been shown that hASCT2 can mediate this process of viral infection. Moreover, we recently found that some viruses of this large interference group can use the related transporter hASCT1 as an auxiliary receptor (36). Specifically, BaEV can use hASCT1 as well as hASCT2, whereas RD114 and type D simian retroviruses cannot use ASCT1 unless it is deglycosylated in its extracellular loop 2 (ECL2). In other studies, we have also found that ECL2 is a critical site for viral utilization of these receptors (M. Marin, D. Lavillette, S. Kelly, and D. Kabat, unpublished results).

In this study, we used both cell-cell fusion and infection assays to demonstrate that HERV-W Env can efficiently use both hASCT1 and hASCT2 as receptors. Furthermore, HERV-W Env also uses the mouse orthologues of these receptors if the N-linked oligosaccharides in their ECL2 regions are eliminated by mutagenesis. This high degree of diversity in receptor recognition is surprising, because no previously studied virus in this interference group can utilize mouse ASCT2 (mASCT2) and because the relevant ECL2 sequences of the mouse and human ASCT1 and ASCT2 transporters have almost no discernible similarity. We infer that HERV-W has evolved to recognize minimal common features in these highly divergent transporters rather than specific features and that its ability to use two proteins as receptors is an adaptive property of this virus family.

MATERIALS AND METHODS

Cell lines. Human embryonal kidney HEK293T cells (ATCC CRL-1573) were grown in Dulbecco's modified Eagle high-glucose medium supplemented with 10% fetal bovine serum (FBS). CHO (Chinese hamster ovary) cells (ATCC CCL-61) and derived clones were grown in Alpha-modified minimal essential medium supplemented with 10% FBS. TE671 human rhabdomyosarcoma cells (ATCC CRL8805) and mouse fibroblast NIH 3T3 cells (ATCC CRL1658) were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS. Clones of CHO cells stably expressing hASCT1, hASCT2, mASCT1, mASCT1.N201T–N206T, mASCT2, and mASCT2.N167H–N230H receptors were made by transfection with previously described *myc*-tagged receptor-expressing vectors (36). Transfected cells were selected with G418, and individual Geneticin-resistant colonies were isolated. For hASCT1, hASCT2, mASCT1, and mASCT1.N201T–N206T, we selected clones that were highly susceptible to infection by BaEV(LacZ) murine leukemia virus (MuLV) vector. For mASCT2 and mASCT2.N167H–N230H, highly expressing cell clones were chosen as determined by Western immunoblotting with a monoclonal antibody specific for the *myc* tag (36). We employ the human and mouse common names, ASCT1 and ASCT2, for the receptor proteins. The standard nomenclature for their genes is SLC1A4 and SLC1A5, respectively (Online Mendelian Inheritance in Man database, National Center for Biotechnology Information, National Institutes of Health [http://www.ncbi.nlm.nih.gov/entrez]).

Plasmids. The phCMV-HERV-W vector expressing the HERV-W envelope was described elsewhere (12). A truncated version of the HERV-W envelope was generated by introducing a stop codon after amino acid 485, leading to a 16-amino-acid-long cytoplasmic tail (A. Ruggieri and F.-L. Cosset, unpublished data). Based on sequence alignment, a fusion-inhibitory peptide R similar to those found in MuLV envelopes (46, 48) was eliminated by mutagenesis from the cytosolic domains of RD114 (V. Sandrin, B. Boson, and F.-L. Cosset, unpublished data) and BaEV envelopes, leading to RD-Rless and BaEV-Rless, respectively. Expression vectors encoding the BaEV-Rless and RD-Rless envelopes were derived from the phCMV-G expressing plasmid (63).

The HIV-1-based vector pHIV-H2-lacZ, with a deletion within the HIV *env* gene and bearing a β-galactosidase-encoding reporter gene, was kindly provided by Richard Sutton (Baylor College of Medecine, Houston, Tex). The pCM-VdR8.74 vector, which encodes the structural proteins of HIV-1 except for the envelope, and pRRLsin18.cPPT.CMV.eGFP.WPRE, which encodes an enhanced green fluorescent protein (GFP)-encoding reporter gene, were kindly provided by Luigi Naldini (University of Turin Medical School, Turin, Italy) (59).

Cell-cell fusion assays. Cells were seeded in 12-well plates at a density of 10^5 cells per well. Twenty-four hours later, cells were transfected with phCMV-RD114-Rless, phCMV-BaEV-Rless, or phCMV-HERV-W by using PolyFect reagent (Qiagen). At 24 to 36 h posttransfection, cells were fixed and stained with May-Grunwald and Giemsa solutions (Merck) according to the manufacturer's recommendations.

Production of lentiviral particles and infection assays. HIV/HERV-W pseudotype viruses were made by cotransfecting human HEK293T cells with the phCMV-HERV-W vector expressing the HERV-W envelope and either pHIV1-H2-LacZ or pCMVdR8.74 plus pRRLsin18.cPPT.CMV.eGFP.WPRE, using PolyFect reagent (Qiagen). At 36 h posttransfection, the viral supernatants were collected and filtered through 0.45-μm-pore-size membranes. Target cells were seeded in 48-well plates at a density of 0.5×10^4 cells per well and incubated overnight at 37°C. Unless otherwise indicated, 250 μl of diluted virus sample containing 8 μg of Polybrene per ml was added to the cells and centrifuged for spinoculation at $1,200 \times g$ for 2 h at 25°C. After removal of the supernatants, the cells were incubated in regular medium for 48 to 72 h at 37°C.

Immunoblot analyses. Six-milliliter samples of virus-containing medium were centrifuged at 30,000 rpm in a Beckman SW-41 rotor for 70 min at 4°C through 2 ml of a 25% sucrose cushion. Viral pellets were resuspended in 100 μl of phosphate-buffered saline (Gibco). Samples (20 μl) were mixed with 4 μl of buffer containing 375 mM Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.06% bromophenol blue and then analyzed by electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS. After protein transfer onto nitrocellulose filters, immunostaining was performed in Tris base-saline (pH 7.4) with 5% milk powder and 0.1% Tween. The blots were probed with the 6A2B2 antibody (12) and then incubated with horseradish peroxidase (HRP)-conjugated immunoglobulins raised against mouse antibody (Dako, Carpinteria, Calif.). Bound enzyme-labeled antibody was visualized using an enhanced chemiluminescence kit (SuperSignal West Pico; Pierce, Rockford, Ill.). The blot was then stripped and reprobed with an anti-HIV p24 mouse monoclonal antibody (hybridoma P23ASG4B9; bioMerieux, Lyon, France). Bound antibody was detected using HRP-conjugated immunoglobulins raised against mouse.

For studies of cell surface expression of mASCT1, mASCT2, and their N-deglycosylated mutants, corresponding CHO clones were surface biotinylated by the addition of 2 mM sulfo-NHS-LC-biotin (Pierce) to 2×10^7 cells for 1 h at 4°C. The reaction was quenched with 20 mM glycine for 15 min. Washed cells were scraped off the culture dishes in cold phosphate-buffered saline and cen-

trifuged at $200 \times g$ and 4°C for 5 min. The cell pellets were then resuspended in lysis buffer (50 mM Tris-HCl [pH 8.8], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, complete protease inhibitor cocktail [Boehringer Mannheim]) and incubated on ice for 30 min. The cell debris and nuclei were removed by centrifugation at $15,000 \times g$ at 4°C for 10 min. The biotinylated molecules were adsorbed onto streptavidin-agarose beads (Gibco BRL) at 4°C. The beads were washed three times with lysis buffer, resuspended in 20 µl of lysis buffer, boiled with an equal volume of $2\times$ Laemmli sample buffer (33). and subsequently analyzed by electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS. The proteins were then transferred to nitrocellulose membranes, which were then treated with 5% milk powder in phosphate-buffered saline. The nitrocellulose blots were probed with anti-*myc* tag monoclonal antibody 9E10 (Sigma) and developed by using an HRP-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc.) and an enhanced chemiluminescence kit (NEN Life Research Products, Boston, Mass.).

RESULTS

HERV-W Env induces cell-cell fusion by interacting with either hASCT1 or hASCT2 transporters. In order to determine its receptor specificity, we transiently transfected a HERV-W Env expression vector into CHO cells that constitutively expressed either hASCT1 or hASCT2, and we analyzed the cultures for syncytium formation. Positive controls were done by using fusogenic derivatives of RD114 and BaEV Env glycoproteins (termed RD-Rless and BaEV-Rless, respectively) from which we had deleted the fusion-inhibitory R peptides from the carboxyl-terminal cytosolic domains (46, 48) and by using human TE671 cells, which are highly susceptible to all of these viruses. Negative controls were done using CHO cultures that were not transfected with Env expression vectors and that lacked hASCT1 or hASCT2.

As shown in Fig. 1, HERV-W Env caused massive syncytium formation in the presence of either hASCT1 or hASCT2, with significantly more extensive syncytium formation occurring in the presence of hASCT1. Although BaEV-Rless also mediated fusions with both receptors, compatible with previous infectivity data (36), the fusion indices in this case were significantly higher with hASCT2 than with hASCT1. Also as expected (36), RD-Rless was able to fuse cells that expressed hASCT2 but was unable to fuse cells that expressed hASCT1. In other experiments we found that expression of the full-length RD114 and BaEV Env glycoproteins in these cells with the same vector did not cause detectable syncytium formation (results not shown). These results confirm the presence of fusion-inhibitory R peptides at the C-terminal extremities of the cytoplasmic tails of these Env glycoproteins. However, the syncytia induced by HERV-W Env were substantially larger than those induced by RD-Rless and BaEV-Rless (Fig. 1), and they also formed more rapidly. Thus, HERV-W Env is highly fusogenic and functionally interacts with both hASCT1 and hASCT2.

Spinoculation and deletion in the cytoplasmic tail of HERV-W Env enhance infectivity of HIV/HERV-W Env pseudotyped viruses. Optimization of HIV/HERV-W pseudotype production was tested as described in Materials and Methods by using two HIV-based vector systems that encode either LacZ or GFP and by cotransfecting these vectors into cell lines in the presence of the phCMV-HERV-W Env expression plasmid. The highest titers of the pseudotyped viruses were obtained using human HEK293T cells, which were also used by An et al. (1), presumably because these cells were more efficiently transfected and because they express these

plasmids at relatively high levels. However, because HERV-W Env causes massive syncytium formation in these cells, maximum pseudotype virus production was obtained by using relatively low quantities of the phCMV-HERV-W Env expression plasmid and harvesting the virions at 36 h posttransfection, prior to extensive syncytium formation. Although the titers of these HIV/HERV-W pseudotyped viruses were consequently fairly low, they were enhanced 30- to 60-fold by spinoculation onto susceptible human cells such as TE671 cells (Table 1). In contrast, spinoculation had no significant effect on the background titers of these same viruses on naturally resistant CHO cells.

Although the factors that limit formation of infectious viral pseudotypes are not fully understood, the length and/or the cleavage site of the cytoplasmic tail of the transmembrane Env component clearly plays an important role (16, 23, 49, 51; Sandrin et al., unpublished data). Consequently, we tested a panel of HERV-W envelopes in which the long cytoplasmic tail was truncated (Ruggieri and Cosset, unpublished data). One of these mutants (termed HERV-Wcyt16), had a cytoplasmic tail that was only 16 amino acids in length (Fig. 2A), and it was more efficient than the full-length wild-type HERV-W Env in generating infectious HIV/HERV-W pseudotypes (Table 1).

In order to better understand the basis for this difference in efficiency of infection, we analyzed the pseudotyped viruses by Western immunoblotting (Fig. 2B) using the 6A2B2 monoclonal antibody directed against the extracellular region of the HERV-W TM protein. In addition, we used gentle conditions for the lysis and electrophoresis in order to preserve oligomeric forms of the TM proteins. As shown in Fig. 2B, the HERV-Wcyt16 TM trimer was much more efficiently incorporated into the pseudotyped virus particles than the wild-type TM trimer. This result, which was reproducibly obtained, suggests that the long cytoplasmic tail of the wild-type Env interferes with efficient processing of the precursor and/or with incorporation of the processed Env glycoprotein into virions. Moreover, the wild-type TM trimer occurs in virions in two forms, one having an apparent M_r of approximately 75,000 and the other being heterogeneous, with an approximate M_r of 53,000 to 60,000. In other studies, we have determined that the smaller wild-type component is a TM trimer with subunits that are partially truncated at their carboxyl-terminal ends by cellular protease(s) (Ruggieri and Cosset, unpublished data). These results are consistent with evidence that Env trimers are necessary for infection (9, 37). Clearly, the structure of the wild-type HERV-W Env is suboptimal for formation of infectious viral pseudotypes. Our interpretations of Fig. 2B are compatible with the expected size differences of the TM trimers and with similar analyses of other HERV-W TM truncation mutants. In addition, Western blot analysis of the corresponding cell lysates showed that they contained equal amounts of proteins (data not shown), supporting our conclusion that the full-length and cyt16 Env proteins are equally expressed and immunoreactive but are differentially incorporated into the virions.

hASCT1 and hASCT2 transporters/receptors mediate infection of HIV/HERV-W pseudotyped viruses. We next asked whether the hASCT1 and hASCT2 transporters can be used as viral receptors when expressed in CHO cells. For these studies we used only HIV-GFP pseudotypes because the HIV-1 long
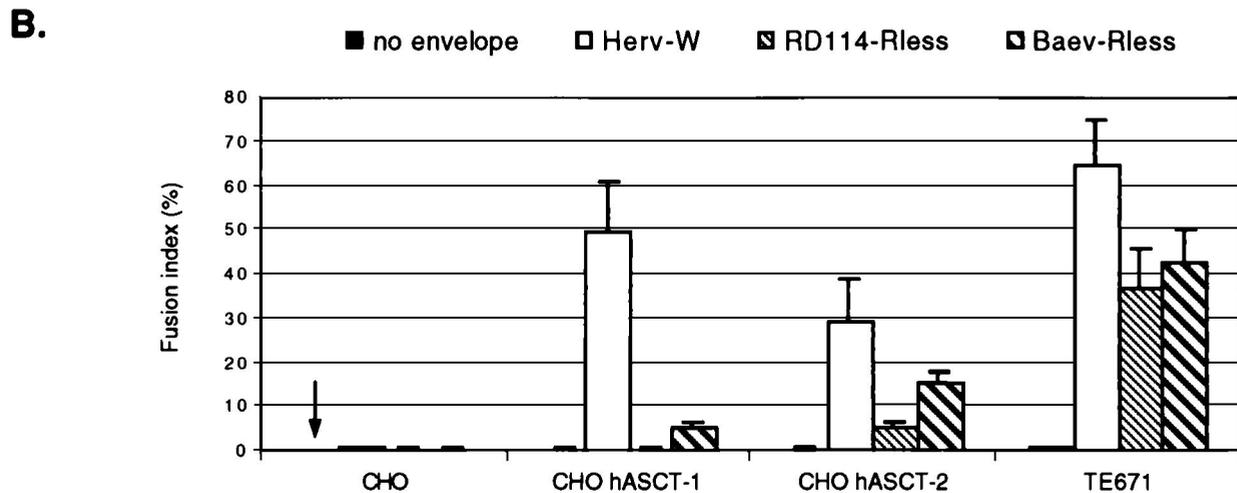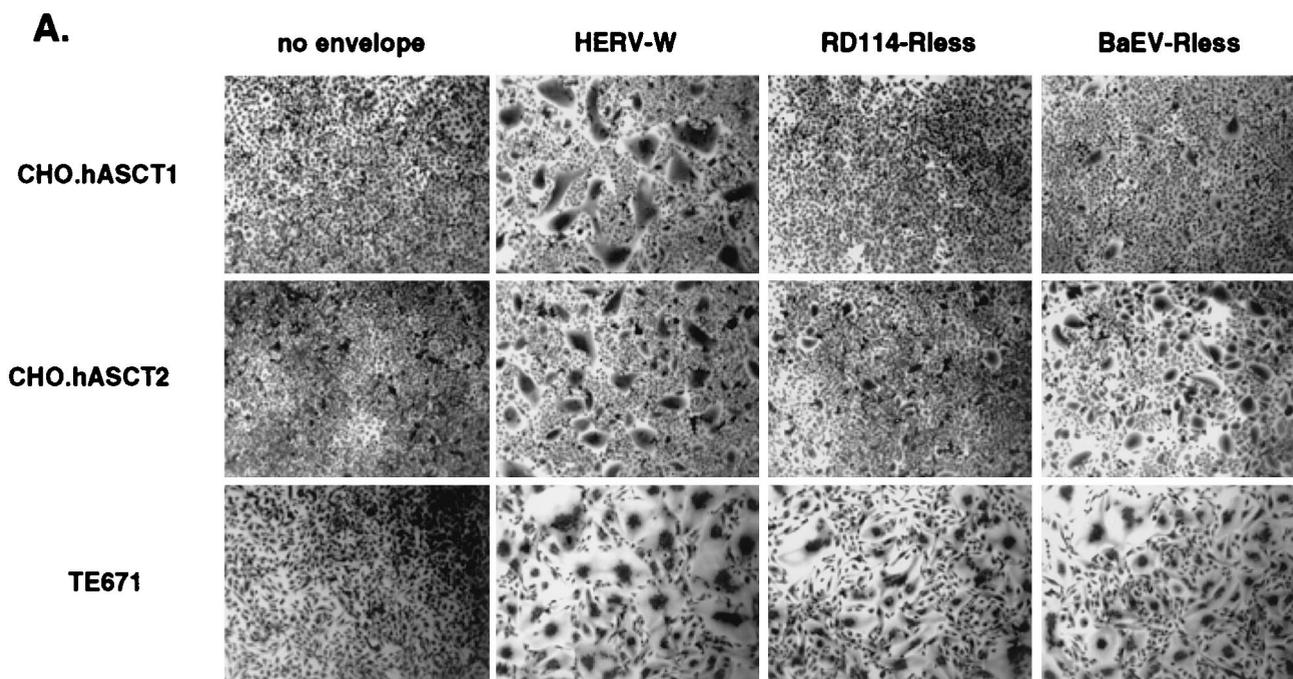
FIG. 1. HERV-W glycoprotein envelope can use both hASCT2 and hASCT1 to induce cell-cell fusion. CHO cells expressing either hASCT1 or hASCT2 and human cell line TE671 were transfected with RD-Rless, BaEV-Rless, HERV-W, or LacZ (control) expression vectors. The determination of the fusion activity of the transfected envelope glycoproteins was performed at 24 h posttransfection. (A) The cultures were fixed and stained with May-Grunwald and Giemsa solutions. Magnification, ×100. (B) Histogram showing the mean fusion index of each combination of cell-cell fusion assay (error bars are standard deviations; $n = 4$). The fusion index represents the percentage of fusion events in a cell population and is defined as $[(N - S)/T] \times 100$, where N is the number of nuclei in syncytia, S is the number of syncytia, and T is the total number of nuclei counted (2).

terminal repeat in the HIV-LacZ pseudotyped vector causes poor expression in CHO cells (10, 22). Accordingly, HIV-GFP virions were pseudotyped with either HERV-W or HERV-Wcyt16 envelopes and used to infect CHO.hASCT1 and CHO.hASCT2 cells by spinoculation. As a control for this analysis, we also examined the susceptibilities of CHO.hASCT1 and CHO.hASCT2 cells to infections by MuLV-LacZ(RD114) and -LacZ(BaEV) pseudotyped viruses. Compatible with our previous evidence that used transient expression of the receptors (36), CHO.hASCT2 cells were

TABLE 1. Spinoculation and deletion of the cytoplasmic tail of HERV-W enhance HIV/HERV-W pseudotype titers[a]

Treatment	Target cell	Titer (CFU/ml)[b] of the indicated virus pseudotypes			
		HIV-LacZ		HIV-GFP	
		HERV-W	HERV-Wcyt16	HERV-W	HERV-Wcyt16
No spinoculation	TE671	1.2×10^3	2×10^4	1.6×10^3	5.2×10^4
	CHO	10	22	16	12
Spinoculation	TE671	7.8×10^4	1×10^5	4.4×10^4	1.6×10^5
	CHO	22	12	40	12

[a] Human TE671 cells were tested for susceptibilities to HIV-LacZ(HERV-W), HIV-LacZ(HERV-Wcyt16), HIV-GFP(HERV-W), and HIV-GFP(HERV-Wcyt16) pseudotype viruses. Hamster CHO cells served as negative control for all of these viruses.
[b] The titers are averages from two independent infection studies

susceptible to both RD114 and BaEV, whereas CHO.hASCT1 cells were highly susceptible to BaEV but were approximately 100-fold less susceptible to RD114 (Fig. 3A). As also shown in Fig. 3A, CHO cells expressing hASCT2 or hASCT1 differed from control untransfected cells in being highly susceptible to viruses pseudotyped with both HERV-W and HERV-Wcyt16 Env glycoproteins. Indeed, these titers were similar to the titers of the same viruses in human TE671 cells. These results strongly suggest that the HERV-W pseudotyped viruses use both hASCT1 and hASCT2 as cell surface receptors.

The mASCT1 transporter and its N-deglycosylated mutant are functional receptors for HERV-W. We previously showed that the mASCT1 transporter is also an efficient receptor for BaEV but not for RD114 or for simian type D viruses (36). However, a mutant form of mASCT1 that lacks N-linked oli-

gosaccharides in its ECL2 region is an efficient receptor for all of these viruses (36). Accordingly, rodent cells treated with tunicamycin were also susceptible to all of these viruses. Based on the fact that HERV-W had a receptor usage similar to that of BaEV in the above-described studies, we determined whether mASCT1 and its deglycosylated mutant would also function as receptors for HERV-W. To address this, we first analyzed NIH 3T3 mouse cells for their susceptibility to infections by HIV/HERV-W pseudotyped viruses. As shown in Fig. 3B, NIH 3T3 cells were weakly but significantly susceptible to both GFP(HERV-W) and GFP(HERV-Wcyt16) infections. Unfortunately, we were unable to determine whether tunicamycin treatment would enhance susceptibility of NIH 3T3 cells to HIV/HERV-W pseudotyped viruses because this treatment followed by spinoculation made the cells inviable. To further
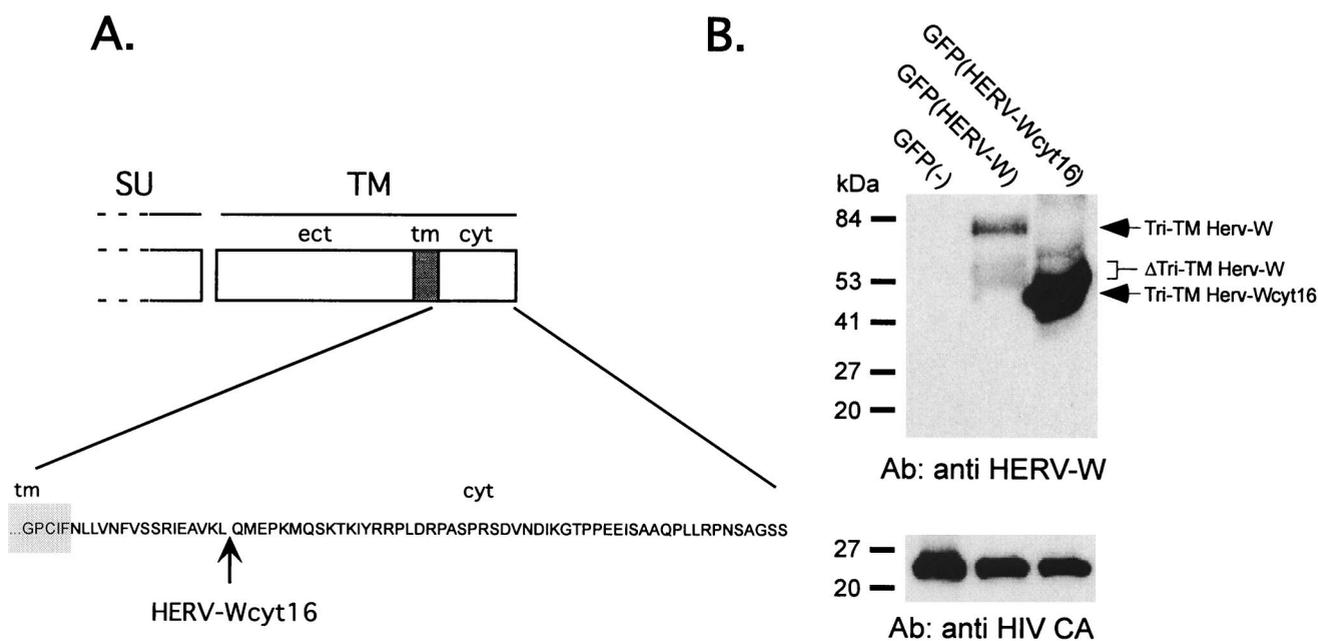

FIG. 2. Analysis of HERV-W and HERV-Wcyt16 envelope glycoproteins in HIV particles. (A) Schematic representation of the carboxy-terminal extremity of the HERV-W envelope. The arrow indicates the stop codon insertion for HERV-Wcyt16. SU, surface subunit; TM, transmembrane subunit; ect, tm, and cyt, ectodomain, transmembrane domain, and cytoplasmic domain of the TM, respectively. (B) Electrophoretic immunoblot of sedimented virus samples probed with monoclonal antibody (Ab) 6A2B2 against HERV-W TM. HEK293 cells were transfected with plasmids expressing HIV Gag-Pol-Rev (pCMVdR8.74), a GFP reporter gene (pRRLsin18.cPPT.CMV.eGFP.WPRE), and a plasmid expressing either no envelope or the HERV-W or HERV-Wcyt16 envelopes. Thirty-six hours later, the supernatants were centrifuged through a 25% sucrose cushion and the pelleted virus samples were analyzed by Western immunoblotting. Normalization of samples was done using an anti-HIV capsid (CA) antibody. Tri, trimer; Δ, truncated form of HERV-W TM.
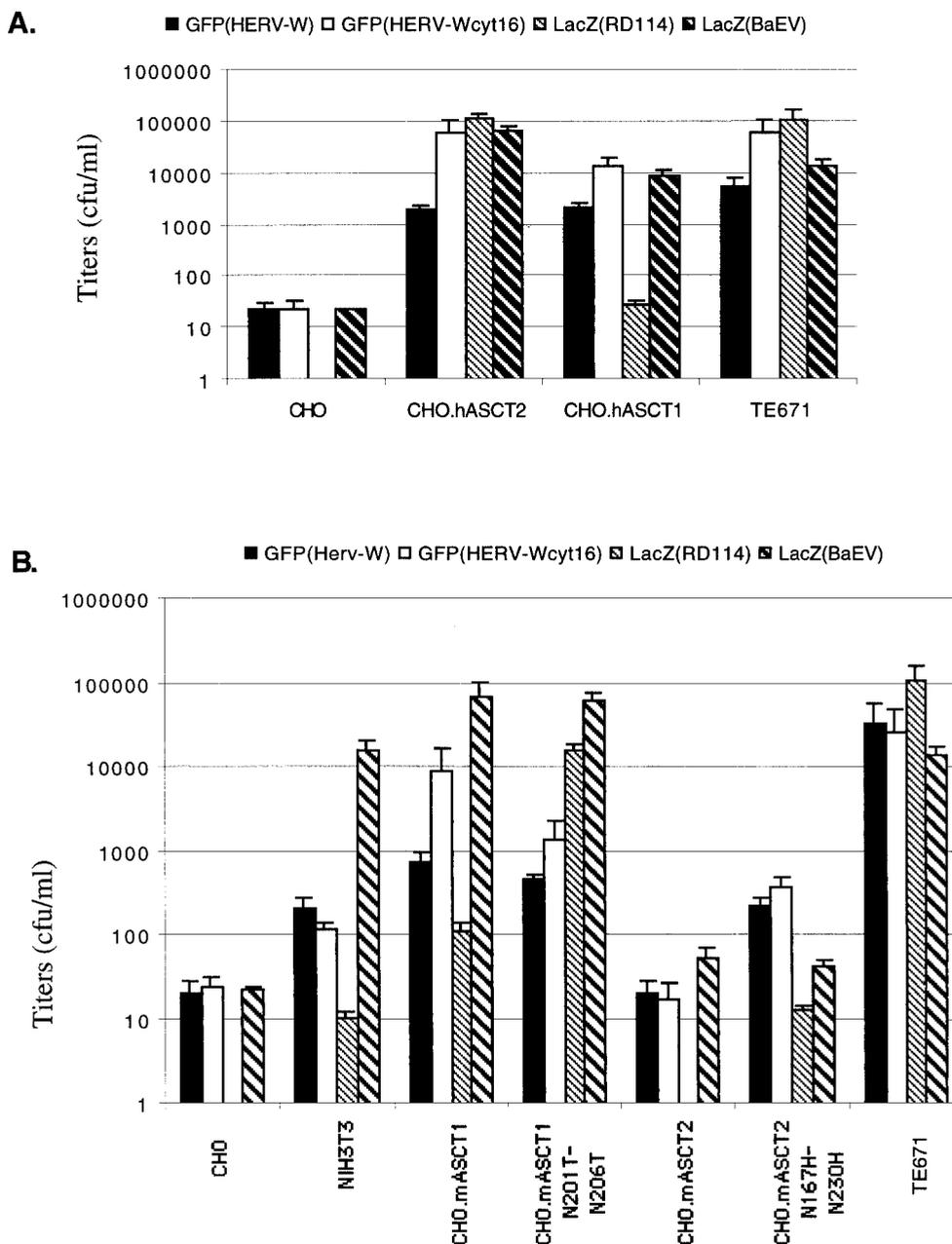
FIG. 3. Mediation of infections by ASCT1 and ASCT2 receptors. Infectivity assays were done using CHO cell clones that constitutively express ASCT1 or ASCT2 receptors. The titers are averages from three independent experiments (error bars are standard errors). (A) Infections of cells that express hASCT1 and hASCT2 receptors; (B) infections of cells that express mASCT1, mASCT2, or their N-deglycosylated mutants.

analyze this issue, we generated CHO cells constitutively expressing either mASCT1 or its deglycosylated mutant mASCT1.N201T–N206T (36). Consistent with our previous results, the CHO.mASCT1 cells were approximately 1,000-fold more susceptible to BaEV than to RD114 pseudotypes, whereas the CHO.mASCT1.N201T–N206T cells were highly susceptible to both of these viruses (Fig. 3B). In addition, both CHO.mASCT1 and CHO.mASCT1.N201T–206T cells were highly susceptible to both HERV-W and HERV-Wcyt16 (Fig. 3B). Similar results were obtained using the cell-cell fusion

assay (Fig. 4). However, in this assay, syncytium formation by the HERV-Wcyt16 envelope in CHO.mASCT1.N201T–N206T cells was somewhat higher than in that CHO.mASCT1 cells.

Since mASCT1 and the double mutant mASCT1.N201T–N206T were tagged at their C termini with the *myc* tag epitope, we analyzed their cell surface expression by using a cell membrane-impermeant biotinylation reagent. Extracts from surface-biotinylated CHO.mASCT1 and CHO.mASCT1.N201T–N206T cell lines were affinity purified by adsorption onto
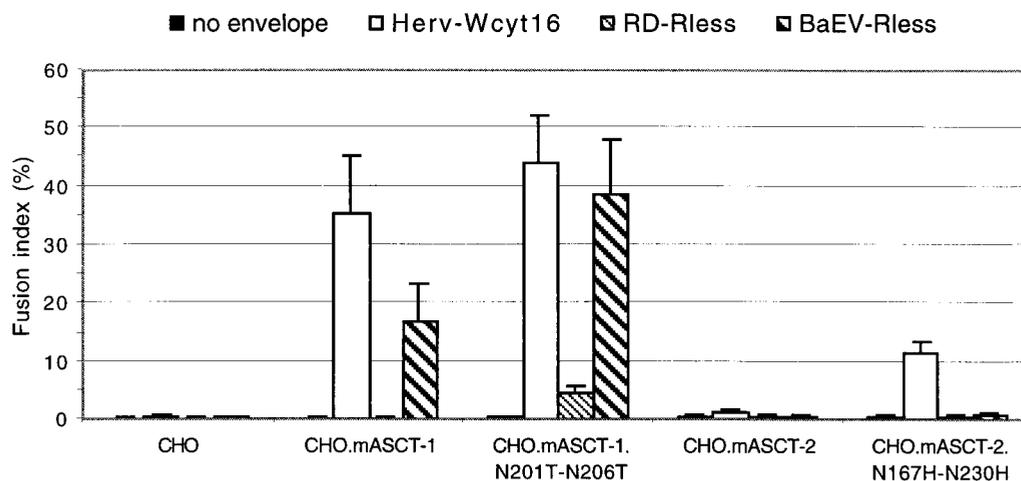
■ no envelope □ Herv-Wcyt16 ◪ RD-Rless ◪ BaEV-Rless

FIG. 4. Fusion assays suggest that the HERV-W envelope glycoprotein functionally interacts with mASCT1 and with N-deglycosylated forms of mASCT1 and mASCT2. CHO cells expressing either wild-type or deglycosylated forms of mASCT1 or mASCT2 were transfected with plasmids expressing the RD-Rless, the BaEV-Rless, or the HERV-Wcyt16 envelopes or the LacZ protein (control). The fusion activities of the transfected envelope glycoproteins were analyzed at 24 h posttransfection. Results are expressed as percent fusion indices (error bars are standard deviations; $n = 3$) as described for Fig. 1.

streptavidin-agarose beads, followed by Western immunoblotting of the biotinylated proteins with the Myc-specific monoclonal antibody (Fig. 5B). As a control for this analysis, we also examined the total protein extracts that had not been adsorbed onto streptavidin-agarose beads (Fig. 5A). The results in Fig. 5B suggest that the wild-type and deglycosylated mASCT1 proteins were biotinylated on the cell surfaces to similar extents. Moreover, we determined that the wild-type and deglycosylated forms of mASCT1 were equally active in amino acid transport (results not shown). Therefore, we conclude that N glycosylation of the mASCT1 protein does not significantly affect its viral receptor function for HERV-W or its processing to the cell surface and activity as a transporter.

HERV-W Env also can employ the deglycosylated form of mASCT2. We previously described evidence that mASCT2 and its N-deglycosylated mutant derivative are inactive as viral receptors despite their significant levels of expression on cell surfaces (36). To investigate their potential activities as receptors for HERV-W, CHO cell clones that constitutively express either wild-type mASCT2 or mutant mASCT2.N167H–N230H, which lacks N-linked oligosaccharides, were generated and selected as described in Materials and Methods. Consistent with our previous results, CHO.mASCT2 and CHO.mASCT2.N167H–230H cells did not mediate infections by either RD114 or BaEV above the level of the CHO cell background (Fig. 3B). However, the CHO.mASCT2.N167H–N230H cells reproducibly showed weak but significant susceptibility to both HERV-W and HERV-Wcyt16 pseudotyped viruses that was approximately 10 to 30% as high as that of the highly susceptible CHO.mASCT1 cells. Moreover, compatible results were reproducibly obtained using the cell-cell fusion assay. As shown in Fig. 4, transient transfection of CHO.mASCT2.N167H–N230H cells with a HERV-Wcyt16 expression vector resulted in a 12% fusion index, whereas no syncytia were observed with either RD-Rless or BaEV-Rless expression constructs. Moreover, expression of the HERV-

Wcyt16 Env glycoprotein in CHO.mASCT2 cells resulted in no visible syncytium formation. To obtain additional evidence pertinent to these results, we analyzed the cell surface expression of mASCT2 and mASCT2.N167H-N230H in CHO cell lines by using the membrane-impermeant biotinylation reagent method. As shown in Fig. 5, the expression of wild-type mASCT2 was approximately 20-fold higher than that of mASCT2.N167H–N230H both in total cell lysates (Fig. 5A) and in the affinity-purified biotinylated samples (Fig. 5B), suggesting that the failure of wild-type mASCT2 to function as a receptor for HERV-W is not due to weak expression on cell surfaces. Conversely, since the deglycosylated mASCT2 protein is expressed on cell surfaces only relatively weakly compared to the wild-type protein, its enhanced ability to mediate infections and syncytium formation with HERV-W Env is highly significant. These studies indicate that the deglycosylated mASCT2 protein functions as a receptor for HERV-W.

DISCUSSION

An important result of the present study is that the HERV-W Env glycoprotein, which has been associated with the human lineage for approximately 25 to 40 million years (28, 29, 60), is able to functionally interact with two highly divergent sodium-dependent transporters of polar neutral amino acids (hASCT1 and hASCT2). Moreover, HERV-W Env not only is able to use the human hASCT1 and hASCT2 transporters but also uses mouse mASCT1 with high efficiency and uses mASCT2 when the latter transporter is N deglycosylated by mutagenesis (Fig. 3 to 5).

Together with previous evidence (36), these results strongly imply that HERV-W is less restricted in its receptor utilization than other retroviruses that use hASCT2 as their common receptor. Indeed, BaEV can use human and mouse ASCT1, but it is unable to use any form of mASCT2. In addition, although RD114 and type D simian retroviruses cannot use
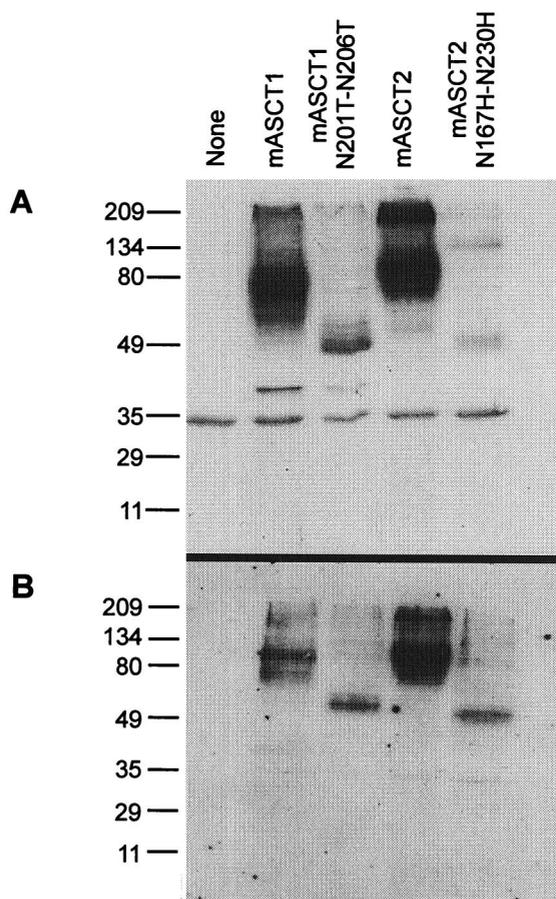
FIG. 5. Identification of mouse ASCT proteins on the surfaces of CHO cells. CHO cells stably expressing *myc*-tagged mASCT1, mASCT2, and their N-deglycosylated mutant proteins were surface biotinylated as described in Materials and Methods. Samples of the total protein cell lysates were also used in a parallel analysis. The biotinylated proteins were then purified by affinity chromatography. The samples were analyzed by Western immunoblotting with anti-*myc* tag monoclonal antibody 9E10 (Sigma). (A) Total cellular *myc*-tagged mASCT1, mASCT2, and their N-deglycosylated mutants proteins; (B) cell surface *myc*-tagged mASCT1, mASCT2, and their N-deglycosylated mutants proteins that were biotinylated on cell surfaces and then affinity purified prior to immunoblot analysis. Numbers on the left are molecular weights in thousands.

native human or mouse ASCT1, they can all use mASCT1 that has been N deglycosylated in its ECL2 region by mutagenesis or by tunicamycin treatment of the cells (36). These and other N-deglycosylation studies as well as studies of human-mouse ASCT2 chimeras have strongly indicated that the ECL2 region of these transporters is critical for their retroviral receptor functions and that other regions may be expendable (M. Marin, D. Lavillette, S. Kelly, and D. Kabat, unpublished results). These results also have clearly established that N-linked glycosylation of ECL2 in ASCT1 plays a critical role in blocking virus-binding sites and in limiting utilization of this receptor by RD114 and type D simian retroviruses (36). The present work substantially extends these studies and shows that HERV-W Env can functionally interact with the mASCT2 protein when it is N deglycosylated. Thus, N-linked glycosylation of ECL2 controls retroviral utilization not only of ASCT1

but also of ASCT2 in some species. It should be noted that the molecular consequence of the N-linked glycosylation of ECL2 still needs to be addressed. It is uncertain whether a bulky N-linked oligosaccharide would only inhibit access of the envelope to ECL-2 or whether it would also mask other loops in proximity to ECL2 in the folded conformation of ASCT proteins. Similarly, although our results strongly suggest that infection by HERV-W requires specific sequences in ECL2, we cannot exclude the possibility that other receptor regions provide secondary sites for envelope interactions. Although there are other examples of viral host ranges that are negatively controlled by N-linked glycosylation of receptors (20, 44, 61), the present example is exceptional because it involves multiple glycosylation sites in two related receptors in distinct mammalian species.

Figure 6 compares the amino acid sequences in the ECL2 regions of the human and mouse ASCT1 and ASCT2 proteins and shows the consensus sites of N-linked glycosylation. Previous results have unambiguously established that all of these consensus sites are indeed N glycosylated and that there are no other sites of N glycosylation in these proteins (36). Interestingly, these four ECL2 sequences are highly diverse, with only 27% amino acid identity, whereas other regions of these ASCT proteins are 55% identical. Moreover, the most critical region of ECL2 for virus interaction occurs in the carboxyl-terminal portion C indicated in Fig. 6 (Marin et al., unpublished results), and this region has only approximately 6% identity, with common features being very difficult to discern. In contrast, ECL2 sequences of members of the glutamate transporter superfamily that are not viral receptors are much more highly conserved (results not shown). These results are compatible with the hypothesis that region C of the ECL2 sequences shown in Fig. 6 has been a battleground for host-virus coevolution in mammals. These interpretations were also strongly supported by analyses of nonsynonymous and synonymous nucleotide sequence changes. For example, we used the SNAP.pl program (32, 40) at www.hiv-web.lanl.gov for comparison of human and mouse ASCT2 nucleotide sequences, and we found a ratio of nonsynonymous to synonymous mutations of 1.56 for region C of ECL2, whereas this ratio was 0.125 for the total proteins and was 0.52 and 0.042 for regions A and B of ECL2, respectively. These results strongly suggest that region C of ECL2 in ASCT2 has been under strong selection pressure to diverge, whereas the nearby central region B of ECL2 has been under strong evolutionary pressure to remain unchanged. It is interesting that the variable region C is adjacent to the highly conserved region B, which presumably is important for normal transporter function (see below).

Usage of multiple receptor proteins would likely be advantageous to a virus for several reasons. First, it would enable the virus to infect multiple tissues that might contribute to its survival and transmission. ASCT1 and ASCT2 transport an overlapping but nonidentical set of polar neutral amino acids, with a major difference being the transport of glutamine only by ASCT2 (4, 15, 27, 58). These transporters also are expressed in overlapping but nonidentical tissues, with ASCT1 being more highly expressed in liver and brain (4). Mouse mammary tumor viruses and mink cell focus-forming viruses provide examples of retroviruses that typically must replicate in different tissues to accomplish their life cycle (13, 17, 53, 62). Presum-
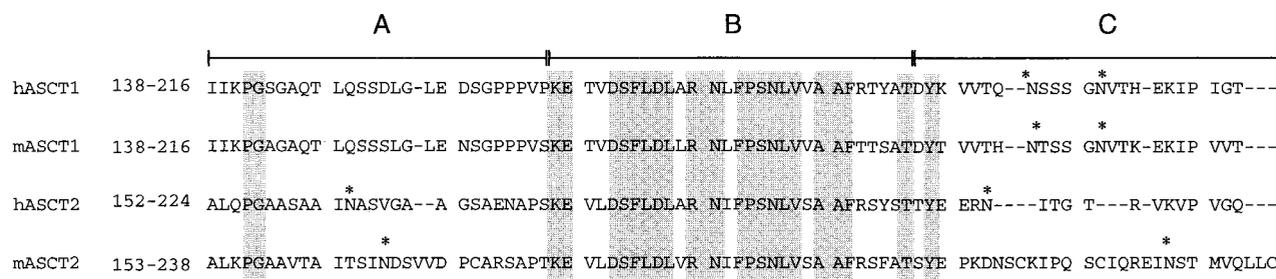
FIG. 6. Amino acid sequence comparison of putative ECL2 of hASCT1, mASCT1, mASCT2, and hASCT2 indicates 27% sequence identity. Numbers at the left of the sequences correspond to the first and last amino acids shown. Common amino acids are shaded. Deletions in sequences are indicated by dashes. N-glycosylation sites are indicated by asterisks. Region C is hypervariable and contains N-linked oligosaccharides that negatively interfere with viral receptor function, whereas region B is highly conserved (see text).

ably germ line infections are likely to be especially important for survival of HERV-W viruses, but it is unclear which of these transporters may occur in such cells. A second major advantage of multiple receptor usages for a virus is that it would substantially prevent selection of host escape mutants. That host escape can occur is strongly suggested by the fact that RD114 and BaEV are xenotropic in their host species (45, 50). However, if a virus uses multiple receptors, mutations in a single receptor would confer a relatively small selective advantage, and such mutations would therefore spread through the host species only slowly and inefficiently, thus providing opportunities for viral adaptations.

Based on the above considerations, we propose that HERV-W and other members of this virus family have been under strong selective pressure throughout evolution to maintain recognition of both ASCT1 and ASCT2 proteins. This hypothesis has at least two important corollaries that appear to be fulfilled for this large virus family. First, this selective pressure would cause the virus Env to recognize common immutable structural features in the highly divergent receptors rather than specific features. In the case of ASCT1, ASCT2, and other members of this glutamate receptor superfamily, there are indeed highly conserved amino acids in region B that occur very near variable region C (Fig. 6). Therefore, we propose that the viruses interact with these conserved sequences in region B and that the hypervariable region C is involved in negatively modulating the conserved viral recognition site(s). A second corollary is that viruses selected to recognize common immutable features of ECL2 in ASCT1 and ASCT2 would also be very likely to have a broad host range, with potential even to jump between species and to cause zoonoses. In agreement with this idea, phylogenetic studies have suggested that HERV-W invaded the human lineage from an unknown and unrelated species approximately 25 to 40 million years ago after the separation of New and Old World monkeys (8, 29). Similarly, there is evidence that RD114 may have originated as a zoonosis in domestic cats and that BaEV may have also invaded the baboon lineage (13). The most striking example of cross-species transmission is the reticuloendotheliosis virus family of avian retroviruses, which is closely related to other members of this virus family, clearly suggesting a rare zoonosis from mammals into birds (5, 25). Further research will be needed to test these ideas and to unambiguously iden-

tify the immutable common sites in ECL2 of ASCT1 and ASCT2 that are recognized by HERV-W and other viruses of this family.

Interestingly, the HERV-W SU glycoprotein is substantially smaller than the SU proteins of RD114 and BaEV. Sequence alignments suggest that the HERV-W SU has a large deletion of a disulfide-bonded variable loop that occurs in RD114 and BaEV SUs. Similarly situated variable loops (termed VR-B) have previously been identified as nonexclusive receptor interaction domains of related retroviruses (6, 7, 54, 55). These considerations imply that the region deleted from HERV-W Env is unnecessary for its recognition of ASCT1 and ASCT2 proteins. It would be interesting to learn whether the corresponding regions of the RD114 and BaEV SU glycoproteins contribute to differences in their receptor specificities.

Although our results confirm earlier evidence of An et al. (1) that the HERV-W Env expressed in placenta can pseudotype HIV-1 virion particles, thus enabling infection of cells that lack CD4, our results suggest that this process is inefficient. In particular, we have found that the long cytoplasmic carboxyl-terminal tail of HERV-W TM hinders its incorporation into HIV-1 pseudotyped virions (Fig. 2) and greatly reduces infectivity (Table 1). Nevertheless, some pseudotyping can occur, supporting the possibility that natural or inflammation-induced HERV-W Env expression might contribute to HIV-1 invasion of otherwise privileged tissues such as the fetus or brain.

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