The infectivity and host range of the ecotropic porcine endogenous retrovirus, PERV-C, is modulated by residues in the C-terminal region of its surface envelope protein

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Abstract

Endogenous retroviral genetic material serves as a reservoir for the generation of retroviral pathogens by recombination between activated endogenous or exogenous infectious agents. Some porcine tissues actively express infectious porcine endogenous retroviruses (PERVs). Of the three classes of PERV characterized to date, two, PERV-A and B, are capable of infecting human cells in vitro, whereas PERV-C cannot. Here, we demonstrate that the PERV-C envelope surface protein (SU) when disassociated from its C-terminus binds human cells. Further, we show that PERV-C binding to human cells is not inhibited in 293 cells productively infected with PERV-A, confirming that the molecule PERV-C interacts with on human cells is distinct from that used by PERV-A. Moreover, we demonstrate that the envelope region encompassing the proline-rich region is required for binding to cells in addition to the putative variable region A (VRA) and B (VRB). The region in the C-terminus of the SU that alters the binding and infectivity properties of PERV-C differs by only nine residues from the analogous region of PERV-A. Caution may be warranted even when a xenotransplantation product is from source pigs that do not express human-tropic viruses, as minimal mutations within PERV-C combined with selection in a human recipient could render PERV-C infectious in humans.

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Endogenous retroviral loci are present in the genome of domesticated pigs and their wild relatives in the Families Suidae and Tayassuidae (Niebert and Tonjes, 2005; Patience et al., 2001). Detailed studies of the LTR structure among these porcine relatives indicate a relatively recent introduction of the porcine endogenous retrovirus (PERV) sequences into the germline, dating to approximately $7.6 \times 10^6$ years ago (Tonjes and Niebert, 2003). Presumably because these are relatively recent additions to the porcine germline, at least some loci present in the pig genome are still encoding infectious retroviruses of the gammaretrovirus genus, but not of other retroviral genera (Patience et al., 2001). The observation that porcine cells express infectious gammaretroviruses poses a safety concern when considering using pigs as source animals for human cell, tissue, and ultimately, organ transplantation. In that context, careful analysis of the viral determinants for human cell tropism is critical.

There are three receptor classes of PERV, named A, B, and C, identified by a combination of sequence divergence, in vitro host range analysis, and classical interference studies (Akiyoshi et al., 1998; Le Tissier et al., 1997; Takeuchi et al., 1998). The isolation of two homologous cDNAs that encode functional receptors for PERV-A, neither of which was functional for PERV-B or PERV-C, confirmed the distinct receptor specificity of these viruses (Ericsson et al., 2003). Of the three receptor classes, only PERV-A and PERV-B are infectious for human cells in vitro. Viruses of the PERV-C class have an ecotropic host range; they are only capable of infecting porcine cells (Takeuchi et al., 1998). However, it has been reported by two
independent laboratories that during in vitro stimulation and culture of primary porcine peripheral blood mononuclear cells derived from NIH mini-swine, viruses with envelope sequences that appear to be derived by recombination between PERV-A and PERV-C may arise that are infectious for human cells (Wilson et al., 2000; Oldmixon et al., 2002). Subsequent analysis suggested that the recombination may occur during the in vitro culture, since sequences bearing these recombinant envelopes could not be found in the genome (Scobie et al., 2004; Wood et al., 2004). In addition, an isolate of human-tropic PERV with a recombinant A/C envelope, PERV-14/220, has been shown to have higher titers on human cells than the “prototype” PERV-A (Harrison et al., 2004). The genetic determinants of the increased infectivity titers of the recombinant envelope were identified in two domains: a single amino acid residue at position 140, and the proline-rich region (PRR) (Harrison et al., 2004).

To date, only limited experimental data exist to identify the genetic determinants of human tropism for PERV. A receptor-binding domain (RBD) for PERV has been inferred to consist of regions analogous to the RBDs identified in homologous gammaretroviruses, such as the murine leukemia viruses (MLVs). The RBD for MLVs has been shown to require the N-terminal 120–200 amino acid sequences of the envelope, depending on the receptor class (reviewed in Overbaugh et al., 2001). The specific structural domains within the RBD that have been shown to be critical for receptor specificity through studies with chimeric envelopes and site-directed mutagenesis have been designated variable regions A and B (VRA and VRB). Interestingly, a third region that shows some variability, the proline-rich region (PRR), is not important for receptor binding for most MLVs, although it is a critical determinant for structural stability and for facilitating the later stages of virus entry (LaVillette et al., 2000; Wu et al., 1998).

No studies of the regions of PERV-C envelope protein required for receptor binding have been performed. Herein, we report the development of reagents and methods for measuring specific binding of soluble PERV-A and PERV-C envelope glycoproteins and results obtained using this assay to identify the regions of the PERV-A and PERV-C envelope that participate in receptor binding. Further, we employed retroviral vectors bearing PERV-A/PERV-C chimeric envelopes and similarly constructed chimeric surface (SU) envelope to examine regions that are critical for human cell tropism by measuring both binding and infection. In these studies, we have identified a region of the PERV-C SU that confers the ability to bind and infect human cells. Finally, we show here that 100 residues at the C-terminus of the PERV-C SU reduce both binding and infection of human cells.

Results and discussion

Construction of soluble epitope tagged PERV-A and PERV-C SU proteins

Based on homology to murine leukemia viruses, regions of the PERV envelope have been identified as putative VRA, VRB, and PRR domains (Le Tissier et al., 1997) (these regions are represented schematically in Fig. 1). We used cDNAs encoding various lengths of PERV-A and PERV-C SUs fused in-frame with the rabbit immunoglobulin G heavy chain (SU-rIgG). PERV-A 200 and PERV-C 200 retain the N-terminal 200 residues of the PERV-A or PERV-C SU, respectively. These N-terminal residues contain the putative VRA and VRB domains, but exclude the PRR. PERV-A 360 and PERV-C 360 retain the N-terminal 360 amino acids of each SU, including VRA, VRB, and PRR domains. PERV-A 460 and PERV-C 440 correspond to the full-length SU. We also constructed two hybrid SU-rIgG proteins, PERV-A/C and PERV-C/A. PERV-A/C contains the N-terminal 333 amino acids of PERV-A, encoding VRA, VRB, and PRR, fused in-frame to the C-terminus of the PERV-C SU. PERV-C/A contains the N-terminal 312 amino acids of PERV-C SU fused in frame to the C-terminus of the PERV-A SU. As a control, we used an SU-rIgG fusion protein derived from the avian leukemia virus (ALV) (Zingler and Young, 1996). Each expression plasmid was transiently transfected into 293T cells and supernatant containing the fusion proteins was harvested 72 h post-transfection, as described in Materials and methods. Immunoblot analysis using the anti-rabbit IgG antibody reacted with the harvested materials demonstrated that all of the fusion proteins were of the appropriate size (Fig. 2). An anti-rabbit IgG ELISA assay was used to standardize the amount of protein used in subsequent assays, as described in Materials and methods.

PERV-A and PERV-C 360 efficiently bind porcine cells

To validate the utility of PERV-A and PERV-C 360 SU-IgGs as functional PERV envelope ligands, we examined the binding characteristics of both PERV-A 360 and PERV-C 360 on a porcine cell line, ST-IOWA using a FACS-based binding assay.
that express the human PERV-A receptor). As shown in Fig. 4, receptors for PERV-A and PERV-C) or SIRC/PAR cells (cells SU-rIgG proteins to bind rabbit SIRC cells (cells that lack receptor (hPAR-2), we compared the ability of PERV-A and C binding analysis of PERV-C 360 SU-rIgG on either SIRC or SIRC/PAR cells. As expected from the results with PERV-C 360, none of the PERV-C-derived SU-rIgG fusion proteins bound either SIRC or SIRC/PAR cells (Fig. 5A). In contrast, PERV-A 360 and PERV-A 460 were able to bind specifically to SIRC/PAR cells, but not control SIRC cells. PERV-A 200 was unable to bind SIRC/PAR cells. These results demonstrate that the N-terminal 200 amino acids, spanning both the VRA and VRB regions of the PERV-A SU are not sufficient to allow binding to cells expressing a functional human receptor for PERV-A and support previous findings that the entire SU region of PERV-A was required for PERV-A to bind its receptor (Watanabe et al., 2005). However, Watanabe et al. did not analyze the receptor binding ability of PERV envelopes where the envelope ligand contained residues that include the PRR yet did not extend to the carboxy terminus of SU, making it difficult to interpret whether the PRR was the sole determinant in the C-terminus of SU required in addition to RBD for PERV-A to bind its receptor. Our analysis of the binding characteristics of PERV-A 360 versus PERV-A 460 SU-IgG fusion proteins allows us to define more specifically the region in the C-terminus of the PERV-A SU that is required, for receptor binding—namely, the PRR.

Our results provide the first demonstration of a specific requirement for the 160 amino acid region including the PRR in binding of PERV-A and PERV-C SU on porcine ST-IOWA cells expressing the functional human receptor for PERV-A (Watanabe et al., 2005). However, Watanabe et al. did not analyze the receptor binding ability of PERV envelopes where the envelope ligand contained residues that include the PRR yet did not extend to the carboxy terminus of SU, making it difficult to interpret whether the PRR was the sole determinant in the C-terminus of SU required in addition to RBD for PERV-A to bind its receptor. Our analysis of the binding characteristics of PERV-A 360 versus PERV-A 460 SU-IgG fusion proteins allows us to define more specifically the region in the C-terminus of the PERV-A SU that is required, for receptor binding—namely, the PRR.

To determine the binding characteristics of soluble PERV-A 360 and PERV-C 360 on cells expressing the human PERV-A receptor (hPAR-2), we compared the ability of PERV-A and C SU-rIgG proteins to bind rabbit SIRC cells (cells that lack receptors for PERV-A and PERV-C) or SIRC/PAR cells (cells that express the human PERV-A receptor). As shown in Fig. 4, binding of PERV-A 360 and PERV-C 360 was not observed on SIRC cells, whereas a marked shift in MFI was observed when PERV-A 360 was incubated with SIRC/PAR cells. In contrast, no shift in fluorescence was observed following FACS-based binding analysis of PERV-C 360 SU-rIgG on either SIRC or SIRC/PAR cells, despite increasing concentrations of soluble SU (Fig. 4). In contrast, for PERV-A 360, a dose-responsive increase in MFI was observed with increasing doses of PERV-A 360. Together, these results demonstrate that PERV-A 360 binding is specific to SIRC cells expressing the human PERV-A receptor. The finding that PERV-C 360 does not bind cells expressing hPAR-2 confirms previous findings that hPAR-2 does not function as a PERV-C receptor (Ericsson et al., 2003).

**Ligands comprising solely the VRA and VRB region of PERV-A fail to bind cells expressing the PERV-A receptor**

Having demonstrated that both the PERV-A 360 and PERV-C 360 SU proteins bind to porcine cells and that PERV-A 360 binds to cells expressing the PERV-A receptor, we next went on to assess more precisely the region(s) of the PERV SU protein that account for their respective receptor specificity. We tested a panel of SU-rIgG fusion proteins (shown in Fig. 1) for their ability to bind SIRC/PAR cells. As expected from the results with PERV-C 360, none of the PERV-C-derived SU-rIgG fusion proteins bound either SIRC or SIRC/PAR cells (Fig. 5A). In contrast, PERV-A 360 and PERV-A 460 were able to bind specifically to SIRC/PAR cells, but not control SIRC cells. PERV-A 200 was unable to bind SIRC/PAR cells. These results demonstrate that the N-terminal 200 amino acids, spanning both the VRA and VRB regions of the PERV-A SU are not sufficient to allow binding to cells expressing a functional human receptor for PERV-A and support previous findings that the entire SU region of PERV-A was required for PERV-A to bind its receptor (Watanabe et al., 2005). However, Watanabe et al. did not analyze the receptor binding ability of PERV envelopes where the envelope ligand contained residues that include the PRR yet did not extend to the carboxy terminus of SU, making it difficult to interpret whether the PRR was the sole determinant in the C-terminus of SU required in addition to RBD for PERV-A to bind its receptor. Our analysis of the binding characteristics of PERV-A 360 versus PERV-A 460 SU-IgG fusion proteins allows us to define more specifically the region in the C-terminus of the PERV-A SU that is required, for receptor binding—namely, the PRR.

![Fig. 3. Binding properties of PERV-A and PERV-C SU-rIgG on porcine ST-IOWA cells. ST-IOWA cells were exposed to increasing concentrations (50–800 ng/ml) of PERV-A and PERV-C SU-rIgG and then incubated for 30 min on ice with anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate. To determine PERV SU-rIgG binding, fluorescence intensity was measured by analyzing 10,000–15,000 live cell events on FACSScan apparatus. The y-axis represents the relative mean fluorescence intensity (MFI) with the highest mean fluorescence corresponding to a value of one. Results shown are representative of three or more experiments. All experiments were done in triplicate.](image-url)
al., 2001). With the exception of one previous report (Faix et al., 2002), regions outside of the classic RBD have not been shown to play a role in expanding receptor utilization properties. Faix et al. (2002) used chimeric FeLV-A/FeLV-B envelope proteins to show that regions C-terminal to the FeLV-B RBD were required for binding certain orthologs of the FeLV-B receptor, Pit1. However, the specific contribution of the PRR region of FeLV-B in virus binding was not examined.

Identification of regions within the PERV-A and PERV-C SU that confer receptor specificity

To assess the regions within PERV-C SU that are required for receptor binding, we examined the ability of the same panel of SU-IgGs to bind porcine ST-IOWA cells (Fig. 5B). Interestingly, neither soluble PERV-A 200 nor PERV-C 200 SU ligands were able to bind ST-IOWA porcine cells whereas both PERV-A 360 and PERV-C 360 efficiently bound to these cells. Thus, similar to the results obtained using PERV-A on SIRC/PAR, we found that both PERV-A and PERV-C require the 160 amino acid region including the PRR for efficient receptor binding.

Regions in the C-terminus of PERV-C SU inhibit binding and infection of human cells

PERV-A but not PERV-C infects human cells such as human embryonic kidney 293 cells (Takeuchi et al., 1998). In order to localize specifically the regions within PERV-A that are important for its human tropism, we assessed binding of PERV-A/C-IgG fusion proteins containing the N-terminal 333 amino acids to various cell types (Fig. 1). As expected, the PERV-A/C SU-IgG was able to bind SIRC/PAR cells (Fig. 5A). It is interesting to note that this ligand composed of the N-terminal 333 residues of PERV-A and residues 334–440 of PERV-C bound both SIRC/PAR and human 293 cells significantly less efficiently than did the PERV-A 360 or PERV-A 460 ligands (P < 0.05) (Figs. 5A and 6A), while there was no significant difference among the binding of these three ligands to ST-IOWA cells (Fig. 5B). These combined observations suggest that there may be sequences in the C-terminal region of the PERV-C SU that inhibit binding to human cells, without effecting binding to porcine cells.

To test this hypothesis, we compared the binding efficiency of PERV-C 360 to PERV-C 440. Similar to PERV-A binding, we found no statistical difference in the MFI measured for PERV-C 360 compared to PERV-C 440 on ST-IOWA cells (Fig. 5B). However, when we examined the relative binding efficiency on human 293 cells, the MFI of PERV-C 440 was significantly reduced compared to PERV-C 360 (P < 0.05) (Fig. 6B), further reinforcing the observation that the C-terminal 100 amino acids inhibits PERV-C SU binding to human cells. The unanticipated finding that PERV-C 360 binds human 293 cells prompted us to rule out non-specific binding of PERV-C 360 to 293 cells. Therefore, we assessed the binding of PERV-C 360, over a range of concentrations and found that the shift in relative MFI corresponded to increasing concentrations of PERV-C 360 (Fig. 6C). These data are suggestive that PERV-C 360 binding to human cells is specific. Similar to our analysis on ST-IOWA cells (Fig. 5B), PERV-C 200 showed only a slight increase in mean fluorescence above
the level detected for the negative control ALV (Fig. 6B) suggesting again the requirement for the region including the PRR to facilitate receptor binding.

The C-terminus of PERV-A SU confers human cell binding and infectivity on PERV-C SU

The finding that binding of PERV-C 440 to human cells is lower than that observed for PERV-C 360 suggests that there are sequences in the C-terminus of PERV-C SU that inhibit SU binding. In order to test whether this may account for why 293 cells are resistant to PERV-C, we assessed the ability of retroviral vectors bearing a PERV-C/A chimeric envelope to bind and infect human 293 cells. As shown in Fig. 6B, PERV-C/A was able to bind human 293 cells to an extent similar to that obtained with PERV-C 360, and the shift in MFI was statistically greater than that for the PERV-C 440 ligand ($P < 0.05$). However, PERV-C/A was not able to bind SIRC/PAR cells (Fig. 5A), indicating that the binding of PERV-C/A to human cells requires a molecule distinct from the PERV-A receptor.

To examine whether the effect of the C-terminus on binding also influences infectivity, we used a retroviral vector pseudo-type composed of MoMLV core proteins and a vector genome encoding beta-galactosidase surrounded by various PERV envelopes. As shown in Fig. 7, all vectors showed comparable titers on ST-IOWA cells; however, vectors bearing a PERV-C envelope were unable to infect SIRC/PAR cells or 293 cells. In contrast, vectors with the PERV-C/A envelope were able to infect 293 cells, albeit at a very low titer. Interestingly, these same vectors were not able to infect SIRC/PAR cells, suggesting that hPAR-2 does not function as a receptor for these vectors. Vectors bearing the PERV-A envelope were able to infect all three cell lines, SIRC/PAR, ST-IOWA, or human 293 cells.
Vectors bearing a chimeric envelope, PERV-A/C, made in an analogous manner to the PERV-C/A SU with the addition of PERV-C-derived sequences in the TM, showed a 100-fold decrease in infectivity titer on human 293 cells compared to PERV-A vectors. These results correspond to the observation that the PERV-A/C SU had decreased binding on human 293 cells, and offers supportive evidence that regions in the C-terminus of the SU of PERV-C negatively impact infection of human cells as well as binding.

**PERV-C binds a receptor present on human cells that is distinct from the PERV-A receptor**

To test whether PERV-C 360 binding to 293 cells can be blocked after infection with PERV-A, we analyzed the relative MFI of PERV-A 360 and PERV-C 360 on 293 cells productively infected with PERV-14/220 (a virus with a PERV-A envelope receptor specificity) (Harrison et al., 2004) compared to uninfected 293 control cells. As expected, PERV-14/220 infected 293 cells exhibited a 60% reduction of PERV-A 360 binding compared to uninfected 293 (Fig. 8A). In contrast, no difference in the level of binding by PERV-C 360 was observed with either infected or uninfected cells. As a control for non-specific effects from 14/220 infection, an SU from Gibbon ape leukemia virus (GALV-RBD) was used. The level of binding observed with GALV-RBD, like PERV-C 360, was similar in either uninfected or infected 293 cells. As a control, we also performed the inverse experiment to compare the relative binding efficiency on uninfected ST-IOWA cells compared to ST-IOWA cells infected with PERV-C. As expected, the PERV-C infected cells inhibited PERV-C 360 binding by over 80% (Fig. 8B). Together, these data demonstrate that the binding and infectivity of PERV-C 360 or PERV-C/A enveloped vectors, are mediated by a receptor that is distinct from that of the PERV-A receptor present on human cells. We have shown that the C-terminal 100 amino acids are critical in modulating the binding and infectivity properties of the PERV-C envelope. There are only 9 amino acid residues which differ between PERV-A and PERV-C in this region (Fig. 9), suggesting that relatively minor genetic alterations of a PERV-C genome may confer human tropism on a naturally occurring PERV-C isolate. The observation that the genetic loci encoding PERV are naturally undergoing recombination events, at least when activated ex vivo (Scobie et al., 2004), will presumably lead to increased genetic diversity of PERV isolates. While it has been proposed that the use of PERV-A and PERV-B free source animals would reduce the risk of PERV transmission to a human recipient of a porcine xenotransplantation product, our results combined with the contribution of ongoing recombination among PERV-C encoding loci suggest that with minimal genetic changes to PERV-C a human-tropic variant of PERV-C may be selected after human xenotransplantation.

It remains to be determined whether the nine residues altered in the C-terminus of PERV-C expand its receptor binding and infectivity properties.

**Fig. 7. Infectivity properties of viral vector pseudotypes bearing PERV-A, PERV-C, PERV-A/C, or PERV- C/A envelopes.**

**Fig. 8. Effect of virus infection on PERV SU-rIgG binding.** (A) 293 cells chronically infected with PERV-A virus, strain 14/420 (293/14220, shown as black bars) as well as uninfected 293 cells (grey bars) or (B) ST-IOWA productively infected with PERV-C or uninfected ST-IOWA cells were incubated with 500 ng/ml of the indicated SU-IgG or GALV-RBD with MFI determined as described in Fig. 3. The y-axis represents the percent binding using the mean value fluorescence intensity of PERV-A 360 binding to 293 cells as 100%. Unstained cells, cells incubated with anti-rabbit IgG conjugated to FITC only, or cells incubated with ALV SU-rIgG were used as negative controls, and GALV-RBD was used as a positive control.
utilization properties by allowing PERV-C to utilize a distinct surface receptor protein or by allowing this virus to employ the human ortholog of the porcine PERV-C receptor. Precedents exist for both. For example FeLV-B, a recombinant retrovirus resulting from recombination of FeLV-A, a feline ecotropic virus, with endogenous sequences within the cat genome, has a broad in vitro host range which includes human cells as well as a wide variety of cells derived from different animal species. FeLV-B utilizes a cell surface receptor, Pit1 that is distinct from the FeLV-A receptor for infection of target cells (Takeuchi et al., 1992). The 10A1 strain of MuLV provides a second example of a gammaretrovirus that arose as a consequence of recombination between the exogenous 4070A MuLV and endogenous retroviral elements and has an expanded host range. The expanded host range of 10A1 results from the ability to use a novel receptor in addition to the 4070A receptor. As few as six residues in the VRA and VRB differ between the 10A1 and 4070A envelopes, and these differences have been demonstrated to account for the expanded host range of 10A1 (Han et al., 1997). To exemplify the second possibility, i.e., that of a recombinant virus utilizing an expanded repertoire of orthologous receptors, it has also been shown that different FeLV-B isolates can employ different Pit1 orthologs for entry: only some FeLV-B recombinants can infect Rat NRK cells and mink cells for example (Faix et al., 2002).

**Materials and methods**

**Cells**

Human embryonic kidney 293 (ATCC 1573), 293T (obtained from Cell Genesys, Foster City, CA), and porcine ST-IOWA (obtained from R. Fister, Tufts University, Boston, MA) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Biosource International, Camarillo, CA) supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml). SIRC cells (rabbit corneal fibroblasts, ATCC CCL-60) and SIRC/PAR, SIRC cells stably expressing human PERV-A receptor-2 cDNA (hPAR-2) (Ericsson et al., 2003), were kindly provided by Dr. Clive Patience (Boston, MA) and were grown in Eagle’s minimal essential medium (EMEM) (Biosource International, Camarillo, CA) supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 U/ml). 293 cells productively infected with PERV-A strain 14/220 were also a kind gift of Dr. Clive Patience. The ST-IOWA cells productively infected with PERV-C were described previously (Takefman et al., 2001). All cells were incubated at 37 °C with 5% CO₂.

**Derivation of PERV env expression plasmids**

PERV-A sequences were derived from a PERV-NIH envelope cDNA originally obtained from activated PBMC from NIH minipigs (Wilson et al., 2000) and introduced into a mammalian expression vector by amplification of PERV-NIH env cDNA using PCR with the following primers: 5’ primer Penv3-SalKoz (5’GTACGTGACGAGATCTTC CACC TGCCACCATGATCCACG3’) and 3’ primer PERVenv4-NotI (5’GTGTGTGGCCCGCCCTTTCATCCCCACCTTC TGTC3’). Penv3-SalKoz introduces a Kozak consensus sequence for optimized eukaryotic translation initiation (Kozak, 1985). The amplified fragment was digested with SalI and NotI, and ligated into pC1neo (Invitrogen, Carlsbad, CA). Sequence analysis was performed using Big Dye Chemistry on an ABI 310 genetic analyzer (PE Biosystems, Foster City, CA).

PERV-C env cDNA was obtained by reverse transcription of RNA isolated from ST-IOWA cells exposed to NIH mini pig plasma as previously described (Takefman et al., 2001). The PERV-C env cDNA was amplified by PCR using the following primers: 5’ primer PervEnvOM2 (5’GAGGCTCTA CTCA- GGAGGAGGACT3’) and 3’ primer PERVenv4-NotI and subcloned into pCR2.1 (Invitrogen, Carlsbad, CA). Identity was confirmed by sequence analysis and compared to previously published PERV-C env sequences (Akiyoshi et al., 1998).

PERV-C env was then amplified by PCR using the Penv3-SalKoz and PERVenv4not1, which allowed the incorporation of
the Kozak consensus signal and a SalI site used for subcloning into pC1neo at the SalI–NotI sites, as described above. The plasmids encoding the entire PERV-A env and PERV-C env are referred to as pC1neoPERVA-1.38.2 and pC1neoPERVC-3.7.2, respectively, throughout the remaining text.

Two chimeric envelope cDNAs were constructed by substitution of a 1000 bp ApaI and SalI fragment from pC1neoPERVC-3.7.2 into pC1neoPERVA-1.38.2 to obtain pC1neoPERVA/C. The resulting pC1neoPERVA/C encode residues 1–333 of PERV-A SU fused in frame to the C-term 334–640 residues of PERV-C envelope. The analogous C/A construct was created similarly by substituting a 1000 bp ApaI and SalI fragment from pC1neoPERVA-1.38.2 into pC1neoPERVC-3.7.2 to obtain pC1neoPERVC/A. pC1neoPERVC/A contains residues 1–312 of PERV-C SU in frame with the residues 312–660 of PERV-A SU/TM.

**Derivation of plasmids encoding PERV SU-rabbit immunoglobulin (PERV SU-rlgG) fusion proteins**

The PERV SU-rlgG fusion proteins PERV-A 360, PERV-C 360, PERV-A 460, PERV-C 440, PERV-A/C, and PERV-C/A SU-rlgG were constructed using pC1neoPERVA-1.38.2 and pC1neoPERVC-3.7.2 plasmids as templates. PERV-A 360 and PERV-C 360 were made by ligating three DNA fragments: (1) a 5461 bp NotI–BamHI fragment of pC1neoPERVA-1.38.2 or pC1neoPERVC-3.7.2, which contains the coding sequences of PERV-A or C SU, respectively, (2) a 2062 bp SalI–BamHI fragment of pC1neo, and (3) a 748 bp BamHI–NotI of pSK100. The pSK100 plasmid (a kind gift of Dr. John Young, University of Wisconsin, Madison, WI) contains the coding sequences for amino acids 96–323 of the rabbit immunoglobulin gamma heavy-chain gene (Genbank accession number P01870) (Zingler and Young, 1996). The resulting plasmids, PERV-A 360 and PERV-C 360, contain residues 1–360 of PERV-A SU and PERV-C 360, contain residues 1–360 of PERV-C SU, respectively, fused in frame to the Ala-96 residue of the rabbit IgG heavy chain coding sequences.

PERV-A 460 was constructed by amplification of a 1416 bp fragment that contains the full-length PERV-A SU envelope sequences using PCR with the following primers: pENV3-SalKoz and PERVASU-reverse2537 (5’ GGACTAGTGGG-CTC TCTTTTTGCC3’). PERV-C 440 was constructed by amplification of a 1322 bp fragment that contains the full-length PERV-C SU envelope sequences using PCR with the following primers: pENV3-SalKoz and PERVCSU-2443 (5’GGACTAGTTTGTGC ATGATTTCCTGATGT 3’). The constructs, PERV-A 460 and PERV-C 440 contain the full-length SU fused to rabbit-IgG.

Plasmids PERV-A/C and PERV-C/A are chimeric envelopes similar to pC1neoPERVA/C and pC1neoPERVC/A except that they encode only the SU portion of ENV and are fused to rabbit IgG. PERV-A/C was created by amplification of PERV-A/C sequences from pC1neoPERV-A/C by PCR using primers pENV3-SalKoz and pENV3-SalKoz and PERVCSU-2443. Similarly, PERV-C/A was created by amplification of PERV-C/A sequences from pC1neoPERV-C/A by PCR using primers pENV3-SalKoz and PERVCSU-2443. The resulting PCR fragment was ligated to pC1neo or pSK100 as described above.

All constructs were confirmed by restriction enzyme analysis and sequence analysis.

**Generation and characterization of PERV SU-rlgG fusion proteins**

To generate the PERV SU-rlgG fusion proteins, 8.5 × 10⁵ 293T cells were plated on 100 mm² petri dishes 24 h prior to CaPO₄-mediated transient transfection using Profection Mammalian Transfection System–Calcium Phosphate(Promega, Madison WI) with plasmids encoding PERV SU-rlgG fusion proteins or a control plasmid encoding the SU-rlgG fusion protein from the avian retrovirus ALSV-A (ALV-rlgG) (Zingler and Young, 1996). Twenty-four hours post-transfection, transfected cells were replenished with DMEM supplemented with 50 μg/ml of leupeptin and 5 μg/ml of aprotinin. Cell culture supernatant was collected 72 h post-transfection, and an additional 50 μg/ml of leupeptin and 5 μg/ml of aprotinin were added to the supernatant and stored at −80 °C for further use. The concentration of SU-IgG fusion proteins was determined by a rabbit-IgG specific enzyme-linked immunosorbent assay (ELISA) by following manufacturer’s directions (Alpha Diagnostic International, San Antonio, TX). To further assess protein expression and stability of PERV SU-rlg G fusion proteins, 1 ml of cell culture supernatant containing PERV SU-rlgG fusion proteins was incubated with 20% protein A-Sepharose beads (Sigma, St. Louis, MO.) for 1 h and protein from immunoprecipitates was assessed by SDS-PAGE and Western blot analysis as described previously (Gemeniano et al., 2003), with the following modification: the nitrocellulose membrane was probed with monoclonal anti-goat IgG linked to horseradish peroxidase. Bands were visualized using Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA) and exposed to X-ray film.

**Assessment for binding of envelope SU-rlgG and SU-HA fusion proteins to target cells**

Binding of PERV SU-rlgG was performed according to methods previously described (Jones et al., 2002; Nath et al., 2003). Briefly, 5 × 10⁵ to 1 × 10⁶ target cells were detached using 0.5 M EDTA, resuspended in 0.2 ml of supernatant containing various concentrations of PERV SU-rlgG as indicated in the results, and incubated for 1 h on ice. The cells were washed twice with cold PBS containing 2% FBS and then incubated for 30 min on ice with anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC) (1:50 dilution) (Jackson ImmunoResearch, West Grove, PA). The cells were then washed twice with cold PBS containing 2% FBS and resuspended in cold PBS containing 4% paraformaldehyde. For experiments involving larger numbers of samples, cells were fixed in 4% paraformaldehyde for 30 min on ice prior to exposure to PERV SU-rlgG. To determine PERV SU-rlgG binding, 10,000–15,000 live cell events were measured for fluorescence on FACScan (BD PharMingen, San Diego, CA).
and analyzed using CellQuest program (Becton Dickinson, San Jose, CA). Data are represented as the Relative Mean Fluorescence Intensity (MFI), whereby each MFI was normalized to the highest mean fluorescence corresponding to a value of one.

The binding of GALV-RBD to target cells was performed similarly, with the exception of incubating target cells with the HA-tagged envelope fragment at 37 °C shaking for 45 min, followed by incubation with anti-HA antibody, HA.11 (Con- vance, Princeton, NJ) at 37 °C for 45 min. The final incubation was with goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch, West Grove, PA). Each incubation with antibody was followed by washing cells with PBS supplemented with 1% FBS, as described previously (Farrell et al., 2002).

**Derivation of viral vector pseudotypes and infectivity assays**

One million 293T cells were seeded in 10 cm² tissue culture dishes 24 h before transfections. Three expression vectors were introduced into 293T cells by CaPO₄-mediated transient transfection using the Profectin Kit (Promega, Madison, WI) (Soneoka et al., 1997; Ting et al., 1998): (1) pRT43.2Tnlsβ-gal (obtained from Tom Dull, Cell Genesys, Foster City, CA) provided the Moloney murine leukemia virus (MoMuLV)-based genome containing the packaging signal and the β-galactosidase gene coding sequence, (2) a MuLV gag/pol plasmid, and (3) pC1neoPERV-A-1.382 or PERVC-3.7.2 envelope plasmids. The resulting pseudovirions are composed of a MoMuLV-derived genome encoding β-galactosidase within a MoMuLV core surrounded by PERV envelope glycoproteins.

Infectivity was determined by histochemical determination of β-galactosidase expression in target cells as previously described (Wilson and Eiden, 1991). All infections were performed in triplicate.

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**References**


