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Comparison of the convergent receptor utilization of a retargeted feline leukemia virus envelope with a naturally-occurring porcine endogenous retrovirus A

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ABSTRACT

In vitro screening of randomized FeLV Envelope libraries identified the CP isolate, which enters cells through HuPAR-1, one of two human receptors utilized by porcine endogenous retrovirus-A (PERV-A), a distantly related gammaretrovirus. The CP and PERV-A Envs however, share little amino acid homology. Their receptor utilization was examined to define the common receptor usage of these disparate viral Envs. We demonstrate that the receptor usage of CP extends to HuPAR-2 but not to the porcine receptor PoPAR, the cognate receptor for PERV-A. Reciprocal interference between virus expressing CP and PERV-A Envs was observed on human cells. Amino acid residues localized to within the putative second extracellular loop (ECL-2) of PAR-1 and PAR-2 are found to be critical for CP envelope function. Through a panel of receptor chimeras and point mutations, this area was also found to be responsible for the differential usage of the PoPAR receptor between CP and PERV-A.

Introduction

Retroviral entry is an intricate process involving coordinated interactions between the retroviral envelope (Env) and a specific host receptor. The expression pattern of this receptor therefore is a major determinant of retroviral tropism. In turn, altering the receptor utilization retargets the retrovirus to cells or tissues that express the alternative receptor(s); a feature that can be exploited to create targeted retroviral vectors for gene therapy applications.

By randomizing the receptor-binding domain of feline leukemia virus (FeLV), novel Envs have been isolated which utilize receptors outside of the FeLV interference group (Bupp and Roth, 2002; Bupp and Roth, 2003; Mazari et al., 2009). This method only alters a small region of the Env and thus does not interfere with its processing or fusogenic properties. Furthermore, the isolation process screens for only functional envelopes (Bupp and Roth, 2002) and little to no background is observed on cells that do not express the viral receptor (Mazari et al., 2009; Sarangi et al., 2007). While this method holds great promise for creating a collection of functional, retargeted Envs, examining the receptor usage of those that have been isolated thus far is vital to fine tuning this system and understanding its full potential.

CP is one such Env isolate derived through this in-vitro system of molecular evolution and selection (Mazari et al., 2009). Its host receptor was isolated and determined to be the same receptor utilized by porcine endogenous retrovirus A (PERV-A) (Ericsson et al., 2003), a distantly related gammaretrovirus that shares low homology to CP (32% identity; 46% homology within SU). This receptor, HuPAR-1, initially reported to function as a G-protein coupled receptor for gamma-hydroxy butyrate (Andriamampandry et al., 2007), has subsequently been characterized as a riboflavin transporter (Yao et al., 2010). PERV-A is also able to utilize a second human receptor, HuPAR-2, also reported to function as a riboflavin transporter with 86.5% sequence identity to HuPAR-1 (Ericsson et al., 2003; Yonezawa et al., 2008).

In this report we have further examined the receptor usage of CP and determined that, similar to PERV-A, CP is able to utilize both of the human receptors, HuPAR-1 and -2, yet is unable to utilize PERV-A's constitutive receptor, the porcine homologue, PoPAR. Unlike the

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human genome, the pig genome appears to carry only a single homologue of PAR that shares similar levels of identity to both HuPAR-1 and HuPAR-2 (approximately 84% in each case) (Ericsson et al., 2003). Furthermore, the data demonstrate that, while CP interacts with a similar region on the receptor surface, the interactions between CP and HuPAR are distinct from those between the receptor and PERV-A. These findings validate the method through which CP was isolated as an efficient means of creating novel and functional Env-receptor pairs.

Results

CP tropism and interference studies

Previous studies of the laboratory-generated CP Env isolate identified the GPR172A gene as the host receptor (Mazari et al., 2009). Remarkably, this protein was previously identified as one of the receptors for the PERV-A virus (HuPAR-1). Virus bearing the CP Env was unable to infect cells of murine origin, a characteristic similar to PERV-A Env (Takeuchi et al., 1998; Wilson et al., 2000). This common receptor usage was striking given the lack of homology between the two Envelopes (Fig. 1). The SU proteins of these two Envs show only 32% identity and 46% homology. Additionally, within the proposed receptor binding domain VRA (underlined region), show only 3 homologues within the entire 36aa domain. Additionally, two specific residues in the PERV-A C-terminal region of SU (Argaw et al., 2008) have been shown to influence human cell tropism. PERV-A V433 lies in a cluster of conserved residues between PERV-A and CP (residue 413 in CP) while R395 in PERV-A, critical for infection of human cells, is a threonine in the corresponding position of CP SU and is found within a domain of amino acids lacking homology between the two envelopes (position 377).

To extend our understanding of the receptor usage of CP and PERV-A, viral titers were measured on a panel of cell lines expressing the human and porcine receptors (Fig. 2, panels A and B), as well as in viral interference studies (Fig. 2, panels A and C). Virus bearing the CP Env was capable of infecting 293HEK cells with titers of 7.3×10^4 *lac*Z staining units (LSU)/ml compared to virus bearing PERV-A, which produced titers of only 2.2×10^3 LSU/ml. Additionally, the CP Env was non-permissive in SIRC cells previously shown to be resistant to PERV-A infection (Ericsson et al., 2003). Introduction of HuPAR-1 into SIRC cells restored viral titers of virus bearing CP Env to levels of 6.3×10^3 LSU/ml, confirming that HuPAR-1 functions as the viral receptor. In the CP receptor isolation, the HuPAR-2 homologue was not identified (Mazari et al., 2009). To analyze the ability of HuPAR-2 to serve as an alternative CP receptor, HuPAR-2 was introduced into SIRC cells. Titers of $>10^4$ LSU/ml were observed on the SIRC-HuPAR-2 cells, for both the CP and PERV-A virus (7.1×10^4 and

```
PERV-A     (1) MHPTLSRRHLPIRGGKPKRLKIPLSFASIAWFLTLSITPQVNGKRLVDSP
    CP     (1) ----------MESPTHPK----PSKDKTLSWNLVFLVGILFTIDIGMANP

PERV-A    (51) NSHKPLSLTWLLTDSGTGININSTQGEAPLGTWWPELYVCLRSVI-----
    CP    (37) SPHQIYNVTWVITNVQTNTQANATSMLGTLTDVYPTLHVDLCDLVGDTWE

PERV-A    (96) P-------GLND--QA-----TPPDVLRAYGFYVCPGPPNNE--E--YCG
    CP    (87) PRYLCRRTGCSWSSKYGCKTTDRKKQQQTYPFYVCPGHAPSLGPKGTHCG
                                    ^
PERV-A   (128) NPQDFFCKQWSCVTSNDGNWKWPVSQQDRVSYSFVNNPTSYNQFNYGHGR
    CP   (137) GAQDGFCAAWGCETTGEAWWKP------SSSWDYITVKRGSSQDNNCEG-

PERV-A   (178) WKDWQQRVQKDVRNKQISCHSLDLDYLKISFTEKGKQENIQKWVNGMSWG
    CP   (180) ----------------------KCNPLILQFTQKGKQ---ASWDGPKMWG

PERV-A   (228) IVYYGGSGRKKGSVLTIRLRIETQMEPPVAIGPNKGLAEQGPPIQEQ---
    CP   (205) LRLYR-TGYDPIALFTVSRQVST-ITPPQAMGPNLVLPDQKPPSRQSQTG

PERV-A   (275) ---R-PSPNPSDYNTTSGSVPTEPNITIKTGAKLFNLIQGAFQALNSTTP
    CP   (253) SKVATQRPQTNESAPRSVAPTTVGPKRIGTGDRLINLVQGTYLALNATDP

PERV-A   (321) EATSSCWLCLASGPPYYEGMARGGKFNVTKEHRDQCTWGSQNKLTLTEVS
    CP   (303) NKTKDCWLCLVSRPPYYEGIAILGNYSNQTNPPPSCLSIPQHKLTISEVS

PERV-A   (371) GKGTCIGMVPPSHQHLCNHTEAFNRTSESQYLVPGYDRWWACNTGLTPCV
    CP   (353) GQGLCIGTVPKTHQALCNKTQQGHTG--AHYLAAPNGTYWACNTGLTPCI

PERV-A   (421) STLVFNQTKDFCVMVQIVPRVYYYPEKAVLDEYDYRYNRPKREPISLTLA
    CP   (401) SMAVLNWTSDFCVLIELWPRVTYHQPEYVYTHFAKAVRFR-REPISLTVA

PERV-A   (471) VMLG----LGVAAGVGTGTAALITGPQQLEKGLSNLHRIVTEDLQALEKS
    CP   (450) LMLGGLTVGGIAAGVGTGTKALLETAQ-----FRQLQMAMHTDIQALEES

PERV-A   (517) VSNLEESLTSLSEVVLQNRRGLDLLFLKEGGLCVALKEECCFYVDHSGAI
    CP   (495) ISALEKSLTSLSEVVLQNRRGLDILFLQEGGLCAALKEECCFYADHTGLV

PERV-A   (567) RDSMNKLRERLEKRRREKETTQGWFEGWFNRSPWLATLLSALTGPLIVLL
    CP   (545) RDNMAKLRERLKQRQQLFDSQQGWFEGWFNRSPWFTTLISSIMGPLLILL

PERV-A   (617) LLLTVGPCIINKLIAFIRERISAVQIMVLRQQYQSPSSREAGR-
    CP   (595) LILLFGPCILNRLVQFVKDRISVVQALILTQQYQQIKQYDPDRP
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Fig. 1. Alignment of CP and PERV-A Env sequences. The SU proteins from CP and PERV-A were aligned using the Vector NTI Alignment Software (Invitrogen). The sequence of the FeLV-A VRA (based on homology to FeLV-B) is underlined (Barnett et al., 2003; Rohn et al., 1994). The 11 amino acid CP specific sequence within VRA selected from the FeLV A/C randomized library is highlighted in bold with an arrow indicating the essential Trp residue (Bupp and Roth, 2002; Rigby et al., 1992). Additionally, the essential PERV-A residues R395 and V433 are highlighted in bold (Argaw et al., 2008).

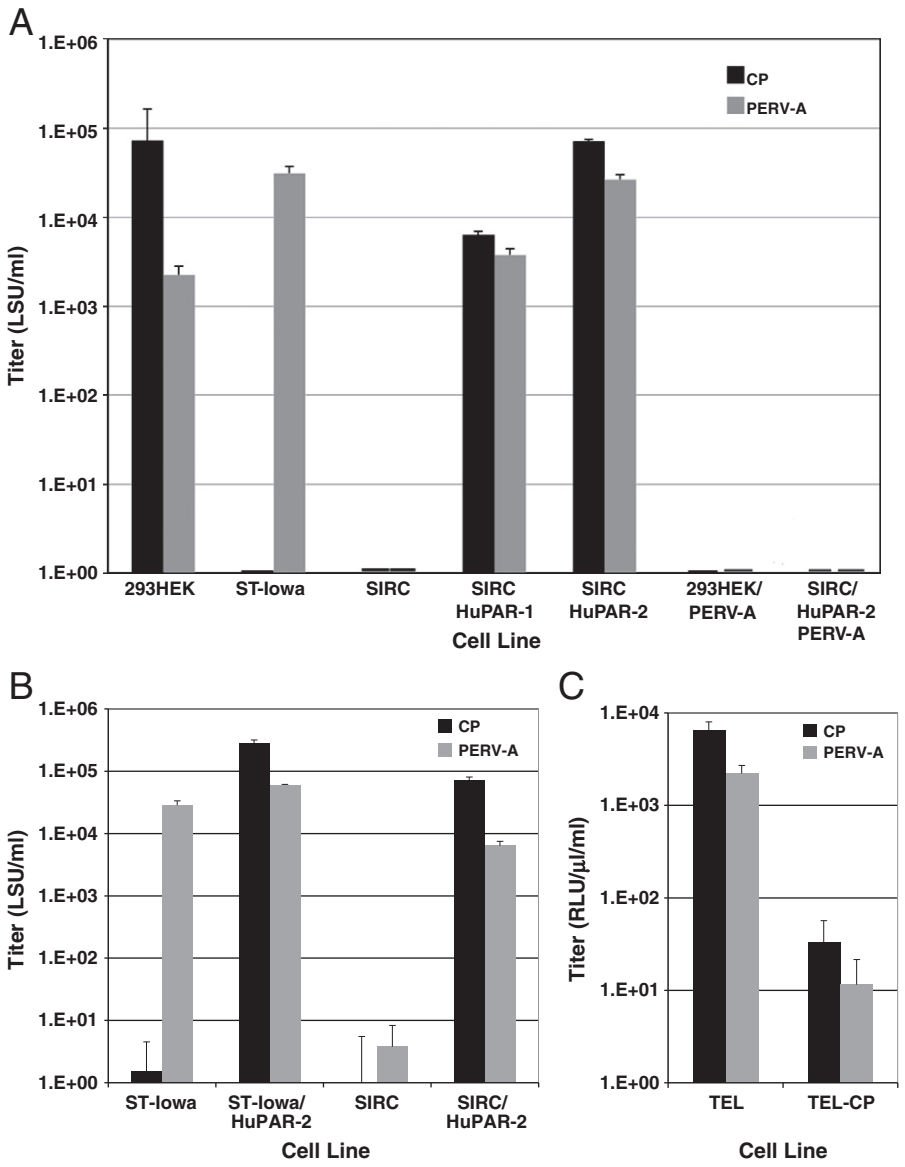

Fig. 2. Interference and receptor usage of CP and PERV-A. (A and B). Viral titer of LacZ staining units/ml (LSU/ml) was determined by the transfer of the *lacZ* marker via retroviral vectors carrying CP (Black) or PERV-A (Grey) Envs on cells expressing either HuPAR-1 or HuPAR-2, as well as on permissive cells productively infected with the PERV-A strain 14/220 (Harrison et al., 2004). The figure represents the average of three (A) or four (B) values with error bars representing one Standard Deviation (SD). (C) Viral titer based on luciferase transfer, RLU/ml of viral supernatant of pseudotyped MLV particles displaying either CP (Black) or PERV-A (Grey) Envs to TELCeB cells or TELCeB cells expressing the CP Env (TEL-CP). The figure represents the average of 3 experiments with error bars representing 1 SD.

2.6×10^4 LSU/ml respectively), indicating that similar to PERV-A, CP is able to utilize this closely related receptor. Lastly, virus bearing the CP Env produced no titer on the ST-Iowa porcine cells, which are highly permissive to PERV-A infection with a titer of PERV-A of 3.1×10^4 LSU/ml. However, expression of HuPAR-2 in ST-IOWA cells was sufficient to allow for high titer infection (2.8×10^5 LSU/ml) by virus bearing the CP env (Fig. 2B) demonstrating that there is no post-entry block in porcine cells but rather that CP is unable to utilize the porcine receptor, PoPAR.

Viruses are capable of blocking infection of a challenge virus when both utilize the same host cell-surface receptor protein, a phenomenon termed receptor interference (Steck and Rubin, 1966). PERV-A virus was therefore introduced into both permissive human 293 HEK cells, and the SIRC-HuPAR-2 cells, and challenged with virus bearing CP Env. In the presence of PERV-A, neither CP nor PERV-A was able to infect either the 293/PERV-A or the SIRC-HuPAR-2/PERV-A cells (Fig. 2A). Additionally, viral titers were measured on cells expressing high levels of the CP Env (TEL-CP) as well as on the parental cell line (TEL) which express no viral Env (Fig. 2C). In this study, firefly luciferase was used as the reporter gene, as these cell lines already express *lacZ*. On the parental TEL cell line, CP and PERV-A had titers of 6.4×10^3 and 2.2×10^3 RLU/ml respectively. Titers were reduced by greater than 100-fold on the CP Env expressing cell line, TEL-CP, yielding CP and PERV-A viral titers of 33 and 12 relative luciferase units (RLU)/ml, respectively. The reciprocal interference between these two Envs confirms their common receptor usage.

HuPAR/MuPAR chimeras and HuPAR-2 mutants

Previous studies have shown that PERV-A is unable to utilize the murine receptor homologue, MuPAR (Ericsson et al., 2003; Mattiuzzo et al., 2007). Through a series of HuPAR-2/MuPAR chimeras and HuPAR-2 mutants expressed in non-permissive SIRC or QT6 cell lines, the block in PERV-A receptor function was localized to a single residue, P109 (Marcucci et al., 2009; Mattiuzzo et al., 2007), which maps to the interface between the second extracellular loop (ECL)

and the fourth transmembrane domain (Supplement 1). This panel of HuPAR-2/MuPAR chimeras and HuPAR-2 mutants was used to survey receptor recognition of CP, shown to utilize HuPAR-2 (Fig. 2A) but incapable of infecting cells of murine origin (Mazari et al., 2009). These data are summarized in Fig. 3A, with the locations of these murine insertions within the HuPAR-2 backbone shown schematically in Fig. 3B. Titers on the permissive cell line 293HEK were 3.9×10^4 LSU/ml. For ten point mutants (H110Y, Q108K, H100N, P97S, Q82R, P73R, V64L, V54L, D40E, and T4P) as well as a triple mutant (VSV(86,88-89)GGI) and an exchange of the entire third ECL (ECL3), the titers were similar to that measured on 293HEK ($>10^4$ LSU/ml). No titer however was detected on cells expressing the L109P mutant, mirroring published data regarding PERV-A titers on cells expressing the same panel of mutant and chimeric receptors (Mattiuzzo et al., 2007).

As antibodies capable of reliably distinguishing between HuPAR-1 and HuPAR-2, or between PARs of different mammalian species have not yet been developed, a surrogate marker was needed to assure proper expression of the non-functional receptor. In these studies, all of the constructs were tagged at the C-terminus with eGFP. Similar levels of fluorescence were measured on cells expressing PAR 2-L109P-eGFP construct as compared to the functional huPAR-2-eGFP construct (Fig. 3C), eliminating concerns of misfolding or rapid turnover of the non-functional mutant. This data highlights the importance of the structure of the second extracellular loop in infection by virus bearing either the CP or PERV-A Envs.

HuPAR/PoPAR chimeras

The most significant evidence for differential receptor usage between CP and PERV-A is found in the inability of CP virus to infect porcine cells, presumably due to a polymorphism in PERV-A's native receptor, PoPAR (Fig. 2A). To better understand this, CP titers were measured on cells expressing HuPAR/PoPAR chimeras in either HuPAR-1 or HuPAR-2 backbones (Fig. 4). The initial construct transferred amino acids 64–235 of PoPAR into HuPAR-2. This region encodes the second and third extracellular loops of PoPAR and contains 49% of the total mismatches between HuPAR-2 and PoPAR and 63% of the mismatches localized within the predicted extracellular loops. This PoPAR insert was further divided into two smaller regions, incorporating residues 62–169 and 169–235 of PoPAR into HuPAR-2.

CP titers on UMR-106 cells transduced with full-length HuPAR-2 were 4.7×10^4 LSU/ml. On cells transduced with HuPAR-2/PoPAR64-169 or HuPAR-2/PoPAR64-235 constructs, no titer was observed. In contrast, expression of HuPAR2/PoPAR169-235 (containing ECL 3 alone) resulted in viral titers (3.7×10^4 LSU/ml) comparable to that of wild-type HuPAR-2. Together, these data place the CP binding site (the site which determines the differential receptor usage of CP and PERV-A) to within aa 64–169. The only predicted extracellular domain within this span is ECL 2.

To further confirm that the region most critical for receptor binding localized within the second ECL, PoPAR 64–169 was exchanged within the HuPAR1 backbone as well. Similarly, while titers on cells expressing HuPAR-1 were 1.1×10^5 LSU/ml, no titer was measured on cells expressing the HuPAR-1/PoPAR64-169. Studies indicate HuPAR1 and HuPAR2 maintain similar topology (Supplemental Fig. S1).

Additionally, binding of virus bearing CP could not be detected on cells expressing the non-functional receptors (data not shown). All three constructs that were unable to mediate CP binding and entry (HuPAR-1/PoPAR64-169, HuPAR-2/PoPAR64-169 and 64–235), contained the second ECL of PoPAR. These data coupled with the data obtained from the MuPAR chimeras demonstrate the critical role that ECL-2 plays in CP binding to the PERV-A receptors.

HuPAR fine mapping studies

To localize the specific residues within ECL-2 that are critical for CP binding and infectivity, CP titers were measured on cells

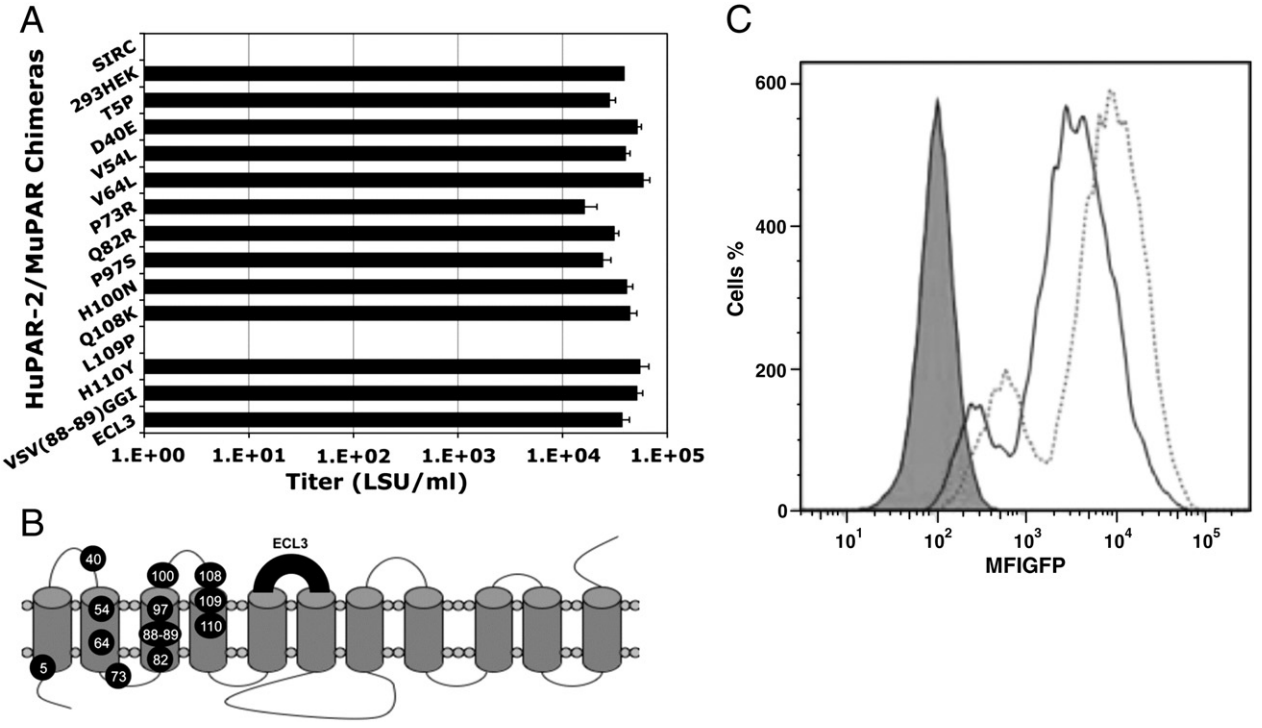

Fig. 3. HuPAR/MuPAR chimeras and HuPAR-2 mutants. (A) Viral titer (LSU/ml) was measured by the transfer of the *lacZ* marker via virus bearing the CP Env into non-permissive SIRC cells expressing a panel of HuPAR-2/MuPAR chimeras. The figure shows the average of three experiments with error bars representing one SD. (B) A schematic representation of the position of the mutated residue(s) within the chimera. Individual point mutations are shown by position. Exchange of the entire ECL3 is shown with a thick black line. (C) GFP expression in SIRC cells and SIRC cells expressing either HuPAR-2 or the HuPAR-2/L109P mutant both of which contained a C-terminal eGFP tag. Solid grey filled, SIRC control cells; solid black line, SIRC cells expressing PAR 2-L109P-eGFP; Dotted grey line, SIRC cells expressing huPAR-2-eGFP.

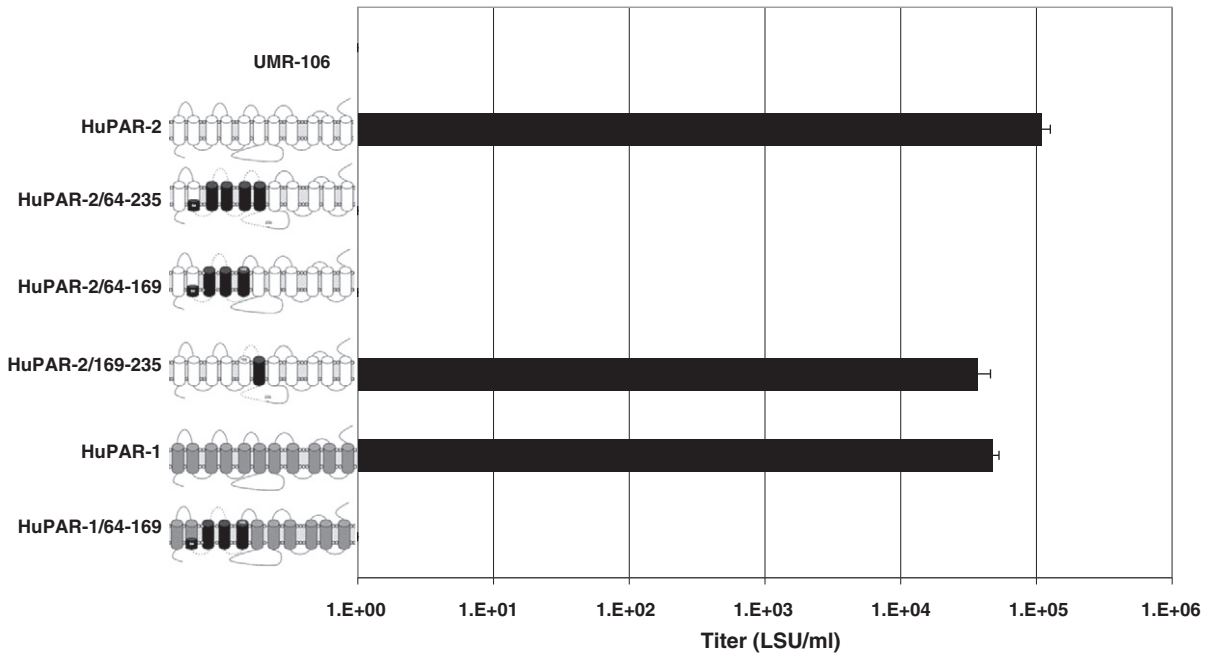

Fig. 4. HuPAR/PoPAR Chimeras. The transfer of the *lacZ* marker by virus bearing the CP Env was measured on non-permissive UMR-106 cells expressing HuPAR-1/PoPAR and HuPAR-2/PoPAR chimeras. The chimeric regions are depicted schematically along the *Y*-axis with the corresponding HuPAR-2 (white), HuPAR-1 (grey) and PoPAR (black) regions represented accordingly. The figure represents an average of three experiments with error bars representing one SD.

expressing a panel of HuPAR/PoPAR chimeras in which residues in the second extracellular loop were mutated alone and in combination. For reference the HuPAR sequence is represented diagrammatically in Fig. 5C with an alignment of the human and porcine ECL's in Fig. 5D. For HuPAR-1, a series of six point mutations and one exchange of PoPAR residues 102–105 were analyzed. For huPAR-2, two point mutations (H100Q, L109V) and exchange of residues VAPV102-105LTVM were generated. In addition, exchange of the entire second extracellular loop (100–109) was generated in both HuPar-1 and HuPAR-2 backbones. Titers on cells expressing wild-type HuPAR-1 and −2 were 1.1×10^5 and 1.2×10^5 LSU/ml respectively. The majority of mutations in HuPAR-1 had little effect on CP titers, with measurements all within 1 order of magnitude of wild-type HuPAR-1 (Fig. 5A). A similar pattern was seen on the HuPAR-2 mutants; however, a greater than 10-fold decrease in titer was seen on the HuPAR-2/L109V mutant (Fig. 5B). For both HuPAR-1 and −2, titers $>5\times10^3$ LSU/ml were maintained until the entire second ECL was replaced with that of PoPAR (mutants HuPAR-1/100-109 (Fig. 6A) and huPAR-2/100-109 (Fig. 5B)). The requirement for such a large change in sequence in order to prevent receptor usage suggests that no single residue is required for recognition by CP but rather virus binding and infection is determined by the overall structure of the second ECL.

Confirmation of chimera expression

In order to assure that the chimeras that CP was unable to utilize were properly synthesized and expressed, PERV-A titers were tested on receptors that were non-permissive to CP (Fig. 6). For the HuPAR2/PoPAR64-235, 64–169, and 100–109 chimeras, PERV-A produced titers of 3.6×10^3, 4.8×10^3, and 1.1×10^4 LSU/ml respectively. These titers were similar to titers measured on cells expressing wild-type HuPAR-2, 8.2×10^3 LSU/ml. Similarly, PERV-A produced titers of 1.3×10^4 and 7.0×10^3 LSU/ml on cells expressing the HuPAR-1 chimeras HuPAR-1/PoPAR 64–235, and 100–109, which were comparable to PERV-A titers on cells expressing wild-type HuPAR-1 (3.8×10^3 LSU/ml). These titers confirm that the receptors were properly expressed and remained functional. Therefore, the lack of CP titers and binding was a result of the chimera itself and not a defect in expression.

Discussion

In this report we have probed the receptor usage of two distantly related gamma retroviruses, PERV-A and CP, which utilize the same human receptors. PERV-A evolved naturally to utilize the porcine receptor PoPAR and fortuitously utilizes the human homologues as well. CP on the other hand, was selected through in-vitro passage and screening of a library of Env constructs on human cell lines. Whereas natural viral evolution is dependent on the presence of other endogenous and exogenous viruses as well as the host immune system, the library screening relies only on functional entry. Other Envs have been developed through the same method as CP which utilize receptors outside of the FeLV interference group (Bupp and Roth, 2003; Bupp et al., 2005). CP is the first however, whose receptor was identified and found to be related to that utilized by a distantly related retrovirus. It was of great interest to compare and contrast the receptor requirements selected by these two independent viruses especially given their striking lack of homology (32% identity and 46% homology) (Fig. 1).

For FeLV-A Env, and thus CP, the major determinant for receptor binding is within the single variable region 1 (VRA) region (Rigby et al., 1992). This region, (residues 82–117 of the CP sequence) shows only 3 homologous residues with PERV-A in the entire 36 aa domain. In contrast, multiple regions of the PERV-A Env are necessary for receptor binding, entry and human cell tropism (Argaw et al., 2008; Gemeniano et al., 2006; Harrison et al., 2004). The ability of PERV-A to recognize PoPAR may therefore be modulated by additional domains of SU that interact with receptor in a way that is unaffected by the structural differences between the human and porcine homologues.

Of interest is the observation that both CP and PERV-A demonstrated ten-fold higher titers on SIRC cells expressing HuPAR-2 than on SIRC-HuPAR-1. This observation has been extensively studied in

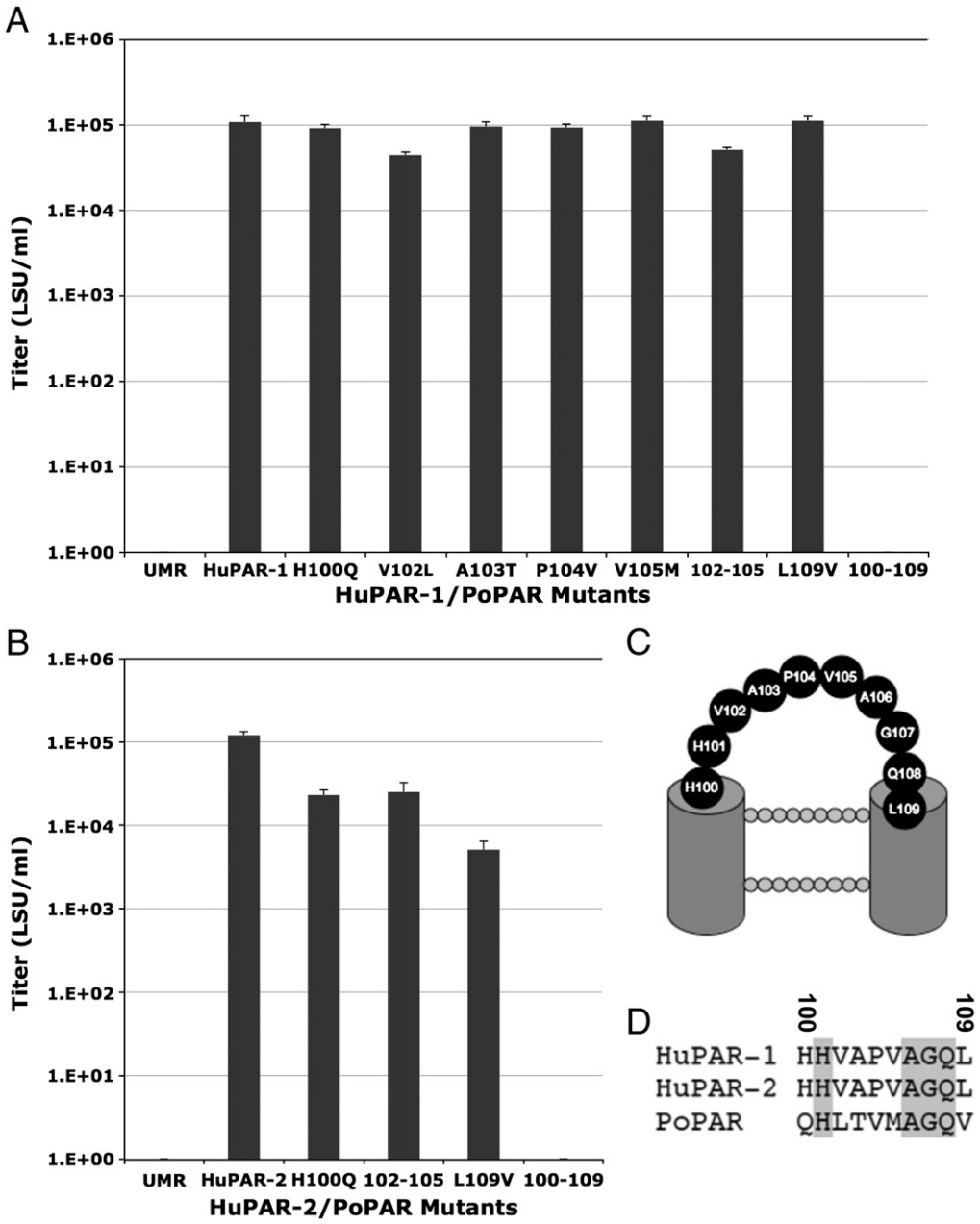

Fig. 5. HuPAR/PoPAR ECL2 mutants. (A and B) The transfer of the *lacZ* marker by virus bearing the CP Env was measured on non-permissive UMR-106 cells expressing a panel of HuPAR-1 (Panel A) or HuPAR-2 (Panel B) mutants in which small domains or single residues of the human sequence had been replaced with the corresponding residue from the PoPAR sequence. The figure represents the average of three experiments with error bars representing one SD. (C) A schematic representation of ECL2 and the location of the mutations in the constructs used in panels A and B. (D) ECL2 sequence alignment from HuPAR-1, HuPAR-2, and PoPAR (residues 100–109). Residues that are identical in all three receptors are highlighted.

PERV-A viral entry studies (Marcucci et al., 2009). Both HuPAR-1 and -2 have been identified as riboflavin transporters (Yao et al., 2010; Yonezawa et al., 2008). However, their expression profile and predicted intracellular domains vary greatly. Interestingly, for PERV-A, chimera studies between HuPAR-1 and HuPAR-2 expressed in SIRC cells indicate the region spanning amino acids 152–285 is responsible for the increase in HuPAR-2 function (Marcucci et al., 2009). This maps outside the major receptor-binding domain (extracellular loop 2), but includes the large third intracellular loop where the most variation between the two proteins map. Thus, receptor properties beyond binding provide advantages for productive infection and high titer. This difference in titer is not seen when the receptors are expressed in UMR-106 cells (Fig. 5). Virus bearing CP binds UMR-106 cells expressing HuPAR-1 and HuPAR-2 at similar levels (Supplemental Fig. S2). This suggests a cell-specific contribution to the relative efficiency with which these two receptors may be used for viral entry.

Data obtained by measuring titers of virus bearing CP on HuPAR-2/MuPAR and HuPAR/PoPAR chimeras demonstrates that like PERV-A, the specific residues located in ECL-2 region are critical for CP binding and infection. This region is identical between HuPAR-1 and −2. In contrast, identity between HuPAR and PoPAR extends to only four of the ten amino acids within this extracellular loop (Fig. 5D). ECL-2 contains primarily hydrophobic residues (Fig. 5C) and may form a hydrophobic pocket on the receptor surface. In a previous study, a tryptophan in the receptor binding region of CP was found to be absolutely necessary for infection, and even conservative mutations rendered the virus non-infectious (Mazari et al., 2009). Perhaps the

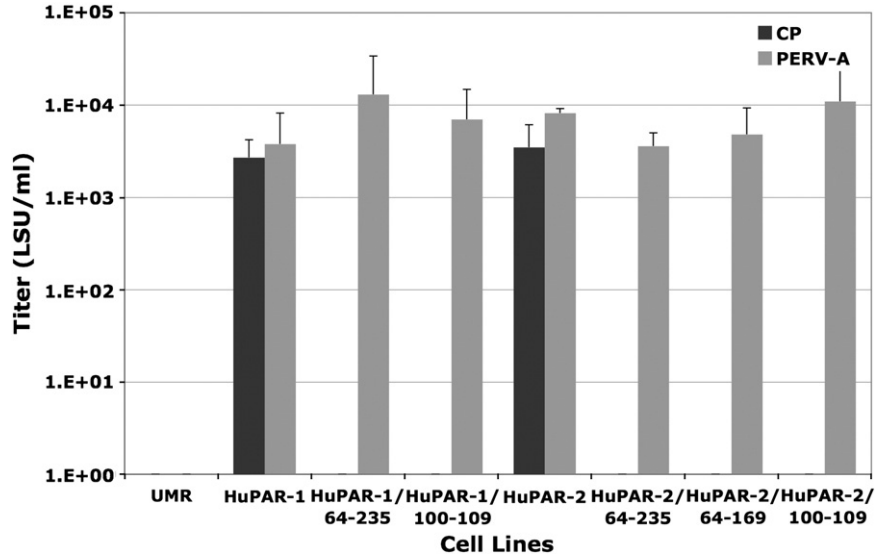

Fig. 6. Confirmation of functional chimeric receptors utilizing PERV-A infection. Viral titer (LSU/ml) was determined by the transfer of the *lacZ* reporter gene via retroviral vectors expressing the CP (Black) or PERV-A (Gray) Env into non-permissive UMR-106 cells and UMR cells expressing the HuPAR/PoPAR chimeras. The figure represents the average of three independent experiments with error bars representing 1 SD.

tryptophan in the VRA of CP inserts into this pocket in a similar but inverse manner to that seen in HIV, in which a single phenylalanine residue on the surface of CD4 interacts with a hydrophobic pocket on the surface of the HIV Env (Kwong et al., 1998). This model is further supported by the ability of CP to utilize the HuPAR/PoPAR point mutants. The majority of the mutations in these chimeras were conservative, replacing one non-polar residue with another (V102L, P104V, V105M, and L109V). These mutants would not be likely to distort this binding pocket sufficiently to prevent the binding of CP. However, when the entire ECL2 is replaced with that of PoPAR, the overall conformation of this hydrophobic pocket may be distorted such that it is no longer accessible to the tryptophan of CP. Definitive proof of this model would be greatly assisted by structural information of both the FeLV A or CP SU and the HuPAR 1 or 2 receptors.

It is interesting that the same HuPAR/MuPAR mutation (L109P) modulates infection by both CP and PERV-A. In rhesus monkeys, cynomolgus macaques and baboons position 109 is a serine and has been also identified as critical for PERV-A viral infection. Substitution of L109S into HuPAR-1 results in loss of PERV-A infection, whereas conversely substituting S109 to L in rhPAR-1 restores PERV-A infection (Mattiuzzo and Takeuchi, 2010). Position 109 is predicted to lie at the junction of the putative second ECL and the fourth transmembrane domain of the human receptor (Supplemental Fig. S1). The substitution of a proline at position 109 may shift this junction, thus altering both the CP and PERV-A binding epitopes. This is supported by the Kyte–Doolittle plot, which predicts the second ECL of MuPAR to be one residue larger and the fourth transmembrane domain to begin after P109. Interestingly, a serine in this position is predicted to have the same effect, positioning residue 109 within the 2nd extracellular domain. This offers a unifying explanation for the critical nature of this position, where PAR receptors non-permissive for CP and PERV-A infection are predicted to encode a 10 amino acid 2nd extracellular loop.

CP was the second Env from our library of mutated envelopes to have its receptor identified. The A5 Env isolate also utilized a multipass transmembrane protein, SLC35F2 (Sarangi et al., 2007), as do the majority of retroviruses. This common evolutionary outcome points to multipass transmembrane proteins as being particularly efficient at providing the features necessary to permit fusion and entry for gammaretroviruses. It is of interest that there are other examples of gammaretroviruses from divergent species that utilize identical multipass transmembrane transporters proteins as their receptors, for example FeLV-B and GaLV Env use Pit1 (Kavanaugh et al., 1994; Olah et al., 1994; Takeuchi et al., 1992). The close proximity of the receptor extracellular domains with the host membrane may provide a spatial advantage for insertion of the viral fusion peptide into the target membrane. Alternatively, the use of a transporter for viral entry may allow viral trafficking to a non-degradative pathway than other cell surface molecules, averting lysosomal degradation (Zhao et al., 1999). Retroviral retargeting is therefore restricted not only by the ability to target the virus to a new cell surface protein (Cosset et al., 1995b; Zhao et al., 1999), but also by the ability to select a target that will be able to function as a retroviral receptor, and allow appropriate intracellular trafficking. The observation that both the natural selection process (PERV-A) and an in vitro-driven evolutionary process (CP) resulted in convergent use of the same receptor, in spite of primary amino acid sequence divergences as demonstrated here, suggest that this is not a result of CP mimicking a distantly related gammaretrovirus, but instead, it is a distinct interaction with two particularly efficient receptors. This validates the method through which CP was isolated as a means of creating truly retargeted Env proteins with novel receptor interaction.

Conclusion

This study illustrates that receptor binding sequences functionally selected in a screen of an in-vitro derived retroviral Env, resulted in convergence of receptor use with a naturally occurring retrovirus. This similar evolutionary endpoint is not a matter of simple molecular mimicry as evidenced by the lack of primary amino acid homology between CP and PERV-A and their differential usage of the porcine receptor. Gammaretrovirus receptor proteins cluster as multipass transmembrane surface proteins. For viruses encoding the CP and PERV-A Envs, their common receptor usage extends not only to the same human receptors but also to a similar extracellular domain. The evolutionary selection for receptor proteins with similar properties highlights the importance of receptor contributions towards mediating efficient viral entry.

Materials and methods

Cell lines

All media was supplemented with 10% FBS and a 10 μl/ml of antibiotic-antimycotic mixture (Gibco). The human renal cell

carcinoma cell lines Caki-1 and -2 were maintained in McCoy's 5A Media; UMR-106 (rat epithelial), 293HEK, and TE671 (human), in DMEM; and SIRC (lapine) and 143B (human), in minimal essential media supplemented with 2 mM L-glutamine. SIRC HuPAR-1 and -2 were maintained in minimal essential media supplemented with 2 mM L-glutamine and 400 μg/ml G418. 293 T were maintained in DMEM supplemented with 400 μg/ml G418. The stable viral producer cell line TELCeB (Cosset et al., 1995a), expressing Gag-Pol plus packaging the *lac*Z, gene was maintained in DMEM supplemented with 10 μg/ml BlasticidinS (Invivogen). TELCeB cells stably expressing the CP Env, TEL-CP (Mazari et al., 2009), as well as 293TCeB cells (Bupp and Roth, 2002) were maintained in DMEM supplemented with 10 μg/ml BlasticidinS and 400 μg/ml G418. UMR-106 expressing the His-tagged receptors and chimeras in pBABE-puro were created by transfecting 5×10^6 293TCeB cells with 5 μg of pHIT-G (Fouchier et al., 1997) (encoding the vesicular stomatitis virus G protein) and 5 μg of plasmid encoding the receptor. Viral supernatant was collected after 48 h and used to infect approximately 5×10^5 UMR-106 cells. The cells were placed into selection with 2 μg/ml puromycin after 48 h. ST-IOWA cells (porcine) were cultured in DMEM supplemented with 10% FBS and 1% Penicillin and Streptomycin, 1% L-glutamine and 1% sodium pyruvate. Sirc Hu/MuPAR cells are lapine cells that stably express chimeric human, HuPAR-2/MuPAR receptors. These cell lines were prepared and maintained as previously described (Marcucci et al., 2009).

Hemagglutinin tagged receptors

Hemagglutinin tags were added to the N or C terminus of the HuPAR-1 cDNA via PCR with KOD Hot Start Polymerase (Novagen). 5′ ATP overhangs were added to the PCR product by incubating 10 μl of the purified PCR product with 10 μl of Taq Master Mix (USB) at 70 °C for 30 min. The products were then Topo-TA cloned into pCRII (Invitrogen). An EcoRI fragment, encoding the tagged HuPAR insert was excised from the pCRII-HA-Par1 and Par1-HA plasmids and exchanged into pcDNA3.1/Zeo(+). 10 μg of the plasmid DNA, isolated using the Endofree Plasmid Purification Kit (Qiagen) was transfected into 293 T cells via Lipofectamine 2000 (Invitrogen). Receptor expression in cells was analyzed 48 h after transfection.

Flow cytometry

All steps were performed in PBS with 5% FBS. Approximately 10^6 cells were harvested with PBS containing 5 mM EDTA, washed with 2 ml wash buffer and incubated on ice for 1 h with the rabbit polyclonal anti-HA antibody HA.11 (Covance) at a 1:100 dilution. The cells were washed and incubated on ice for 45 min with an FITC conjugated polyclonal goat anti-rabbit IgG (Sigma) at a 1:100 dilution. After an additional wash, fluorescence was measured on a Beckman Coulter Cytomics FC500 at the Cytometry Core Facility at the Environmental and Occupational Health Sciences Institute of the University of Medicine and Dentistry of New Jersey.

Binding assay

Virus bearing the CP Env was harvested from a confluent plate of TEL-CP cells and 4 ml was applied to approximately 10^6 UMR-106 cells expressing either HuPAR-1 or HuPAR-2 on ice for 45 min. Virus bound to the cell surface was measured as previously described (Kadan et al., 1992).

Viral Titers

Approximately 10^6 TEL-CP cells were plated into a 10 cm^2 plate containing 10 ml of media in the absence of Blasticidin S or G418. Viral supernatant was collected 48 h later, filtered through 0.45 μm filters, brought to a final concentration of 8 μg/ml polybrene, and applied to cells. After 3 h, the viral supernatant was removed, the cells were washed with PBS, and fresh media was applied. After 72 h titers were measured as previously described (16). For infectivity assays with PERV-A, 293 cells productively infected with the PERV-A isolate 14/220, kindly provided by Clive Patience, were transduced with VSV-G pseudotyped MLV vectors carrying the RT43.2TnIsbgal plasmid, a murine leukemia virus-based vector genome containing the packaging signal and the β-galactosidase gene (Ting et al., 1998) to generate PERV-A 14/220 LacZ (hereafter referred to as PERV-A). Supernatants were collected from confluent monolayers of 293/PERV-A 14/220 LacZ producer cells, filtered through 0.45 mM filter, adjusted to 8 μg/ml polybrene and added to target cells, as described above. Detection of infected cells was performed by histochemical staining and microscopic enumeration of beta-galactosidase-positive cells, as described previously (Wilson and Eiden, 1991).

For measurement of PERV-A titers on the HuPAR/PoPAR chimeras, approximately 3.5×10^5 TELCeB cells (Cosset et al., 1995a) were plated in 10 cm dishes. The next day the cells were transfected with 7 μg of pClneoPERV-Aenv1.38.2CW expressing the PERV-A Env (Takeuchi et al., 1998; Wilson et al., 2000) using the Lipofectamine 2000 kit according to the manufacturers protocol. The next day the cells were induced with 10 mM sodium butyrate for 6 h. 48 hours later virus was harvested and used to infect the target cell lines as above.

Luciferase based viral interference assay

A bicistronic MMLV-based vector designated pCPILW was generated, with CP expression driven by the LTR and *luciferase* gene driven by an IRES (X. Zhang, personal communication). A derivative viral construct lacking the Env gene cassette was used as negative control (pdEnvILW). PERV-A-pseudotyped retroviral particles were produced by the cotransfection of PERV-A vector (pCIneoPERV-Aenv1.38.2CW) and RR460 vector (gift of Dr. Allen Rein (NIH), expressing the *luciferase* gene from the MuLV LTR). The positive control cotransfected pHIT-G (Fouchier et al., 1997) expressing VSV-G with the Env^- vector. 293TCeB cells were transfected either with pCPILW (10 μg) vector, PERV-A (10 μg) plus RR460 vector (5 μg), pdEnvILW (10 μg) or VSV-G (5 μg) plus pdEnvILW vector (10 μg), using the Lipofetamine 2000 transfection kit (Invitrogen), as recommended by the manufacturer. One day after transfection, cells were incubated with 10 mM of sodium butyrate at 37 °C for 6 h. Viral supernatant was collected 48 h post-transfection and filtered through a 0.45 μM filter. 2×10^5 viral targeting cells, TELCeB and TELCeB/CP, were infected with each viral supernatant in either 1 ml or 4 ml. Each transduction was repeated in triplicate. The cells were incubated with viral particles overnight followed by luciferase assay 48-h post-transduction.

For the luciferase assay, transduced cells were collected and lysed in 150 μl cell lysis buffer using the Luciferase Assay System Kit (Promega). Cell lysate (5, 10 and 20 μl) were then added to 100 μl substrate buffer and measured immediately with a GloMax 20/20 luminometer programmed to perform a 2-second measurement delay followed by a 10 second measurement of luciferase activity. The final reading was adjusted as relative luciferase units (RLU) per μl cell lysate per ml viral supernatant (RLU//ml).

HuPAR/MuPAR mutants

HuPAR-2 mutants containing MuPAR substitutions were created and expressed in the non-permissive cell line SIRC as previously described (Marcucci et al., 2009).

HuPAR/PoPAR chimeras

HuPAR-1 and -2 constructs with N-terminal His_6 tags were generated via PCR with KOD polymerase and inserted into pGEM-T

(Promega). The PoPAR cDNA from positions 192 to 706 (aa 64–235) fused in frame with a BamHI restriction site at the 3' end was synthesized (GeneScript) and used as a template. The BstEII–XhoI, XhoI–BamHI, and BstEII–BamHI fragments, encoding amino acids 64–169, 169–235, and 64–235 respectively, were exchanged into His-HuPAR-2-pGEM-T. The full-length chimera was then exchanged into pBABE-puro (Addgene). The BstEII–Xho fragment was also exchanged into HuPAR-1 in the same fashion.

HuPAR-1 and -2 mutants were created via overlapping PCR with KOD polymerase. The final product was gel isolated and purified, digested with EcoRI and BamHI for HuPAR-1 or EcoRI and SalI for HuPAR-2 and inserted into pBABE-puro. All mutations were verified by sequencing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.virol.2012.02.012.

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