Identification of receptors for pig endogenous retrovirus

Thomas A. Ericsson*, Yasuhiro Takeuchi†, Christian Templin*‡, Gary Quinn*, Shelli F. Farhadian*, James C. Wood*, Beth A. Oldmixon*, Kristen M. Suling*, Jennifer K. Ishii§, Yoshinori Kitagawa¶, Takayuki Miyazawa†¶‖, Daniel R. Salomon§, Robin A. Weiss†, and Clive Patience*,****

*Immerge BioTherapeutics, Inc., 300 Technology Square, Cambridge, MA 02139; †Wohl Virion Centre, Windeyer Institute, University College London, 46 Cleveland Street, London W1T 4JF, United Kingdom; §Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; ¶Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; and ‖Host and Defence, PRESTO, Japan Science and Technology Corporation, Tachikawa City, Tokyo 565-0871, Japan

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Xenotransplantation of porcine tissues has the potential to treat a wide variety of major health problems including organ failure and diabetes. Balanced against the potential benefits of xenotransplantation, however, is the risk of human infection with a porcine microorganism. In particular, the transmission of porcine endogenous retrovirus (PERV) is a major concern [Chapman, L. E. & Bloom, E. T. (2001) *J. Am. Med. Assoc.* 285, 2304–2306]. Here we report the identification of two, sequence-related, human proteins that act as receptors for PERV-A, encoded by genes located on chromosomes 8 and 17. We also describe homologs from baboon and porcine cells that also are active as receptors. Conversely, activity could not be demonstrated with a syntenic murine receptor homolog. Sequence analysis indicates that PERV-A receptors [human PERV-A receptor (HuPAR)-1, HuPAR-2, baboon PERV-A receptor 2, and porcine PERV-A receptor] are multiple membrane-spanning proteins similar to receptors for other gammaretroviruses. Expression is widespread in human tissues including peripheral blood mononuclear cells, but their biological functions are unknown. The identification of the PERV-A receptors opens avenues of research necessary for a more complete assessment of the retroviral risks of pig to human xenotransplantation.

A serious donor-tissue shortage is a major barrier to clinical therapies that might be used to treat severe illness such as end-stage organ disease and diabetes mellitus. In this context, appropriate animal donors have the potential to be a renewable and unlimited source of tissues for transplantation that could also be transgenically engineered to enhance their efficacy and safety. However, the promise of clinical xenotransplantation is offset at the present time by the potential of a public health risk due to the cross-species transmission of pathogens from animal donors to human patients (1, 2).

Particular attention has been focused on the risks associated with porcine endogenous retroviruses (PERVs). This attention reflects the current opinion that the pig is a suitable species to engineer as the source for many types of organs and tissues. In the context of a possible infectious disease risk, we have reported previously that multiple copies of PERV are contained in all pig genomes (3, 4).

Two subgroups of PERV (PERV-A and PERV-B) can infect human cells *in vitro*, and these subgroups use cellular receptors that are distinct from those used by each other and other retroviruses (3–5). We sought to identify the receptor used by PERV-A, because all human-tropic PERV isolates obtained from primary pig cells have been PERV-A (6, 7), and this subgroup of virus is present in porcine DNA at a higher copy number than PERV-B (4). Thus, it is likely that PERV-A represents the primary PERV subgroup for which humans are at risk in the context of pig-tissue xenotransplantation.

The viruses related most closely to PERV [gibbon ape leukemia virus, feline leukemia virus, and murine leukemia virus (MLV)] are associated with hematopoietic cell malignancies (8, 9). Therefore, if PERV transmission were to occur, the risk to the transplant recipient and possibly the general populace might be real, especially if the initial infection were to occur under conditions of intensive immunosuppression that might allow time for the virus to adapt to infection of human tissues. Accordingly, to determine the nature of the risk and maximize safety in the design of clinical xenotransplantation trials, it is critical that the biology of PERV be understood. The identification of the molecules that PERV uses as receptors to infect human cells represents a significant step toward this goal.

Methods

Cell Lines, Virus Stocks, and Tropism Studies. SIRC cells (rabbit corneal fibroblasts, ATCC CCL-60) were grown in Eagle's minimal essential medium supplemented with 10% FBS. HeLa (human cervical epithelial cells, ATCC CCL-2), 293 (human embryonic kidney epithelial cells, ATCC CCL-1573), PK15 (pig kidney, ATCC CCL-33), and the retroviral pseudotype cell lines (TELCeB-derived) (5) were grown in DMEM supplemented with 10% FBS. Sf-9 insect cells were grown in TC-100 medium (Sigma) supplemented with 10% FBS; High-Five insect cells (Invitrogen Life Technologies, Carlsbad, CA) were grown in Express-five serum-free medium (Invitrogen Life Technologies), both at 27°C.

Retrovirus infections were performed as described (3) in the presence of 8 μg/ml polybrene. As appropriate, PERV infection was detected by five assays: (*i*) G418 selection, (*ii*) β-galactosidase (LacZ) staining (10), (*iii*) measurement of reverse transcriptase (RT) activity in culture supernatants with an ELISA-based system optimized for PERV RT (HS Mn kit, Cavidi Tech, Uppsala), (*iv*) PCR for PERV *env* with the primers 5′-CCTACCAGTTATAATCAATTTAATTATGGC-3′ and 5′-AGGTTGTATTGTAATCAGAGGGG-3′ (94°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec), or (*v*) real-time quantitative PCR. For real-time PCR assays, cells were challenged with PERV-A 14/220 supernatant for 24 h followed by culture for 48 h at 37°C in fresh culture medium. DNA was isolated from the cells by using the QIAamp DNA mini kit (Qiagen, Valencia, CA). The DNA was quantitated, and 1 μg of DNA (≈50,000 cell equivalents) were subjected to real-time PCR (iCycler, Bio-Rad) by using 1 unit of AmpliTaq gold (Perkin–Elmer Biosciences), 200 μM dNTPs, 3.0 mM $MgCl_2$, and 5 pM of each

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Abbreviations: PERV, porcine endogenous retrovirus; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell; MLV, murine leukemia virus; HuPAR, human PERV-A receptor; BaPAR-2, baboon PERV-A receptor 2; EGFP, enhanced GFP; PoPAR, porcine PERV-A receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY070774 (HuPAR-1), AY070775 (HuPAR-2), AY070776 (BaPAR-2), and AY134475 (PoPAR)].

‡Present address: Leibniz Research Laboratories for Biotechnology and Artificial Organs, Hannover Medical School, D-30659 Hannover, Germany.

**To whom correspondence may be addressed. E-mail: clive.patience@immergebt.com or r.weiss@ucl.ac.uk.

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PERV *pol*-specific primer 5′-AGCTCCGGGAGGCCTACTC-3′ and 5′-ACAGCCGTTGGTGTGGTCA-3′ along with 5 pM of 5′-FAM-CCACCGTGCAGGAAACCTCGAGACT-BHQ-3′ probe. Cycling parameters were 50°C for 2 min, 95°C for 10 min, 60 cycles of 95°C for 15 sec, and 60°C for 1.5 min. Test samples were compared with a standard curve generated from 10^1 to 10^7 copies of plasmid containing PERV *pol* DNA.

The following viruses were used for this study. (*i*) Replication competent PERV: The replication-competent PERV-A 14/220 was isolated in 293 cells after their infection by PERV released from peripheral blood mononuclear cells (PBMCs) of an inbred miniature swine (7). This culture contains two PERV-A species, both of which possess the *env* receptor binding domains of PERV-A in combination with the remaining *env* sequences of PERV-C (GenBank accession nos. AF417227 and AF417228). Replication-competent PERV-B was isolated in 293 cells after their exposure to PK15 cell supernatant (5). (*ii*) Replication-competent PERV-A pseudotypes: PERV-A encoding G418 resistance (PERV-A 14/220/Neo) was produced by the transduction of 293 PERV-A 14/220 cells with the MLV-derived packagable pLN vector (A. D. Miller, Fred Hutchinson Cancer Research Institute, Seattle) (11) and selection in G418. Minimum toxic concentrations for G418 for untransfected mammalian cells were determined over a 14-day period with subculture as necessary to maintain the cells in a subconfluent state. In a similar fashion, PERV-A stocks encoding β-galactosidase (LacZ) activity (PERV-A 14/220/LacZ) were produced by the transduction of 293 PERV-A 14/220 cells with the MLV-derived LacZ reporter vector MFGnlsLacZ (10). (*iii*) Replication-defective PERV-A pseudotypes: Virus supernatants encoding LacZ activity were produced in the TELCeB packaging cell line (5). This cell line produces pseudotyped retrovirus particles comprising MLV Gag-Pol proteins and the LacZ reporter vector MFGnlsLacZ. PERV-A, -B, and -C envelope proteins were incorporated into the particles via stable expression of the Env protein in the TELCeB cells as described (5), producing PERV-A/LacZ, PERV-B/LacZ, and PERV-C/Lac-Z pseudotypes, respectively.

Infection interference assays were used to determine the receptors used by the viruses present in the PERV-A 14/220 isolate and performed in duplicate or triplicate. 293 cells, either uninfected or preinfected by PERV-A 14/220 or biological clones of PERV-A PK or PERV-B PK (5) were used as target cells. These cells were challenged with LacZ pseudotypes of PERV-A 14/220, PERV-A, PERV-B, or PERV-C.

Receptor Cloning. Approximately 2×10^6 subconfluent SIRC cells were transduced with a pantropic HeLa cell cDNA retroviral library (multiplicity of infection ≈3) in accordance with manufacturer instructions (BD Biosciences CLONTECH). The cells were cultured for 48 h before being exposed to PERV-A 14/220/Neo for 6 h (multiplicity of infection ≈5) and then expanded to 25% confluency. After 48 h the cells were subjected to G418 selection until resistant colonies developed. The library-encoded cDNA [human PERV-A receptor (HuPAR)-1, GenBank accession no. AY070774] was isolated from an infectable SIRC colony by PCR with HotStarTaq polymerase (Qiagen) and the primers 5′-AGCCCTCACTCCTTCTCTAG-3′ and 5′-GATGTTTGGCCGAGGCGG-3′ (95°C for 10 sec, 55°C for 45 sec, and 72°C for 180 sec). The HuPAR-2 coding sequence (GenBank accession no. AY070775) was amplified from an oligo(dT)-primed 293 cell cDNA (Superscript System, Invitrogen Life Technologies, Carlsbad, CA) by using Optiprime PCR buffer 2 (Stratagene) and the PCR primers 5′-CCAAAGCATCTTTGGACCTACC-3′ and 5′-TCACGATGAAGACAGGTGGG-3′ (95°C for 10 sec, 55°C for 30 sec, 72°C for 90 sec). The baboon PERV-A receptor (BaPAR-2, GenBank accession no. AY070776) and the murine homologue (GenBank accession no. AK008081) were amplified by using nested PCRs from oligo(dT)-primed cDNAs prepared from testes cDNA by using the following primers and conditions: mouse PCR first round, 5′-GVCTGTKACCTTYGCYYTG-3′ and 5′-RCAAAYCMCAYRDAGGTCYCAG-3′; mouse second round 5′-GTKACCTTYGCYYKWCCTGG-3′ and 5′-CTGGSTCYRRGCCTGSTC-3′; baboon PCR first round, 5′-GTKACCTTYGCYYKWCCTGG-3′ and 5′-GGAGYKGGGTCCCCACCTG-3′; baboon second round, 5′-AATGGCAGCACCYMCGC-3′ and 5′-TCAGGGGCCACAGGGGTC-3′ (95°C for 10 sec, 55°C for 30 sec, and 72°C for 120 sec). The porcine PERV-A receptor (PoPAR, GenBank accession no. AY134475) was amplified from an ST-IOWA cell oligo(dT)-primed cDNA by using the primers 5′-AATGGCAGCACCCACGCYSGSCCG-3′ and 5′-GGCTCAGRRCYCACASKGGTC-3′ with PCR conditions of 95°C for 10 sec, 50°C for 30 sec, and 72°C for 90 sec. PCR products were cloned into Topo-pCRII (Invitrogen Life Technologies) and sequenced by using a Beckman CEQ2000 and associated reagents. Sequence alignments were constructed by using the VECTOR NTI program (InforMax, Frederick, MD).

For receptor-function studies, either the *Not*I–*Not*I fragment (for HuPAR-1) or the *Eco*R1–*Eco*RI fragment (for all remaining receptors and AK008081) of the Topo-pCRII clones were subcloned into the pcDNA3 mammalian expression vector and stably transfected into cell lines by using Lipofectamine PLUS (Invitrogen Life Technologies) followed by continuous selection in G418. Pseudotype infection assays indicated that single-cell clones derived from these bulk G418-resistant cultures did not vary markedly in their susceptibility to PERV infection.

Construction of Tagged HuPAR Receptors. To generate an enhanced GFP (EGFP)-tagged C-terminal HuPAR-2 fusion protein (HuPAR-2/EGFP), the HuPAR-2 ORF was amplified from the Topo-pCRII clone by using the primers 5′-ACGCGGTACCCAGGGGTCTACACAGTCCTTT-3′ and 5′-ACGCAGATCTAGCATCTTTGGACCTACCTAG-3′, which contain *Kpn*I and *Bgl*II restriction sites. The product was cloned into Topo-pCRII (Invitrogen Life Technologies) and excised by using *Kpn*I and *Bgl*II. This fragment was cloned upstream and in-frame of the EGFP ORF in the *Kpn*I and *Bgl*II fragment of the EGFP fusion vector pEGFP-N1 (BD Biosciences CLONTECH) and transfected into SIRC cells.

To generate an N-terminal His-tagged HuPAR-1 receptor protein (HuPAR-1/His), the *Bam*HI and *Eco*RI fragment of pCRII–HuPAR-1 was excised and ligated into pcDNA6HisB encoding 6×-HIS and Xpress epitopes to the N terminus of the expressed protein (Invitrogen Life Technologies). Purified plasmid DNA was linearized with *Bgl*II and purified by gel electrophoresis. SIRC cells were transfected with 2 μg of linearized N-labeled HuPAR-1-containing plasmid by using a calcium-phosphate method (12). Stable transfectants of SIRC were selected in 50 μg/ml blasticidin.

Cellular Localization Assays. Confocal microscopy analysis was performed on sections of SIRC HuPAR-2/EGFP (see below) mounted in Slow Fade medium (Molecular Probes) and viewed on a Zeiss Axiovert 35M microscope equipped with a laser scanning confocal attachment (MRC-1024, Bio-Rad) with an oil immersion 40 × 1.3-numerical aperture objective lens. Fluorescent images were collected by using the EGFP filter set with an argon/krypton mixed-gas laser. Images were generated by using Adobe PHOTOSHOP 5.5 (Adobe Systems, Mountain View, CA).

Receptor Transcription Assays. Expression of the PERV-A receptors was examined by Northern blot and RT-PCR. Northern blots were performed on primary human tissue blots (BD Biosciences CLONTECH) by using a ^{32}P-labeled probe generated by PCR from a HuPAR-1 DNA template and the primers 5′-CCCAGTGGCAGGACAGTTG-3′ and 5′-TCAGCGCGTTGGTGGC-3′ (95°C for 10 sec, 55°C for 30 sec, and 72°C for 60 sec). All lanes contained comparable levels of RNA as determined by probing for β-actin. RT-PCR was performed by using random primed cDNAs generated from the PBMCs of 11 healthy volunteers with the primers and

Table 1. PERV-A tropism and receptor utilization: receptor interference

Target cell lines	Approximate LacZ pseudotype titer, units/ml		
	PERV-A 14/220 LacZ	PERV-A LacZ	PERV-B LacZ
293	1000	400	200
293 PERV-A PK	20	<4	200
293 PERV-A 14/220	<4	<4	200
293 PERV-B PK	1000	200	<4

Interference studies show that PERV-A 14/220 uses the same receptor as prototype PERV-A PK isolate. 293 target cells, either uninfected or infected with replication-competent PERV-A or PERV-B, were exposed to LacZ pseudotypes of PERV-A and PERV-B. A >10-fold reduction in titer indicates receptor blocking.

5′-AAAGCGTGTCTGGCCCTAGG-3′ and 5′-CCTGGAGGC-CTGATCCTAAC-3′ (HuPAR-1); and 5′-CCAAAGCATCTTT-GGACCTACC-3′ and 5′-GATCCCAGTTGAAGTTCAGGC-3′ (HuPAR-2) (95°C for 10 sec, 55°C for 30 sec, and 72°C for 60 sec).

PERV Envelope-Binding Studies. His-tagged envelope fragments containing the VRA and VRB regions for PERV-A and PERV-B were expressed in a recombinant baculovirus system. Binding was analyzed by flow cytometry (see *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org, for a full description).

Results

Identification of PERV-A Receptors. We used a cDNA-library approach to identify a human PERV-A receptor. Briefly, a human cDNA library derived from cells permissive to PERV infection was introduced into a rabbit cell line that is nonpermissive for PERV infection. Cells that had been rendered infectable by the introduction of the library were identified by challenging the bulk cell population with a drug-selectable PERV-A pseudotype. The library-encoded cDNA was cloned, and its activity as a receptor was confirmed by using several approaches.

We began by surveying a series of cell lines for infection by an isolate of PERV-A that we derived from cocultures with mitogen-activated miniature swine PBMCs, PERV-A 14/220. This isolate uses the same receptor as prototype PERV-A isolates from PK15 cells but has a higher titer on human cell lines (Tables 1 and 2). This increase in titer of PERV-A 14/220 may be due to recombinations that have occurred within the *env* gene outside the receptor-binding domain, because alterations in these regions have been shown to affect the infectious titers of other gammaretroviruses (13).

We first determined whether cell lines were infectable by this PERV-A. A series of subconfluent candidate cell lines from a selection of species were challenged with PERV-A 14/220/Neo (titer $\approx 10^5$ tissue culture 50% infective dose per ml in 293 cells) followed by G418 selection after 48 h on the premise that resistance to G418 would be delivered to any cells infected by PERV. Interestingly, using this methodology we found that several human, nonhuman primate, and rodent cell lines that were thought to be uninfectable by PERV-A were susceptible to infection by this high-titer PERV-A stock, suggesting that PERV-A may be able to infect more cell types and species than was reported originally (5) (data not shown).

Table 2. PERV-A tropism and receptor utilization: receptor specificity

Target cell line	Approximate LacZ pseudotype titre, units/ml			
	PERV-A14/220	PERV-A	PERV-B	PERV-C
SIRC (rabbit)	<4	<4	49	<4
SIRC HuPAR-1	4×10^3	6	59	<4
SIRC HuPAR-2	2×10^4	1×10^3	36	<4
SIRC BaPAR-2	6×10^3	NT	NT	NT
SIRC PoPAR	1×10^3	2×10^4	29	<4
SIRC AK008081	<4	<4	NT	NT
NIH3T3 (mouse)	<4	<4	NT	NT
NIH3T3 HuPAR-1	8×10^3	16	NT	NT
ST-IOWA (pig)	8×10^4	1×10^4	19	1×10^3
293 (human)	8×10^4	1×10^4	127	<4
HT1080 (human)	4×10^4	20	NT	NT
HOS (human)	2×10^3	<4	NT	NT

HuPAR-1, HuPAR-2, BaPAR-2, and PoPAR are functional PERV-A receptors when expressed in SIRC and, for HuPAR-1, in NIH3T3 cells. NT, not tested.

During the screen described above, we found the rabbit cell line SIRC to be completely resistant to PERV-A infection and therefore chose this line for use in the receptor-cloning experiments. We transduced SIRC cells with a HeLa cell cDNA library based on evidence that these human cells are readily infected by PERV-A (5). Library-transduced SIRC cells were challenged with the pseudotyped PERV-A 14/220/Neo particles and G418 selection. Surviving cells (i.e., PERV-infected) were cloned and then rescreened by using a LacZ pseudotype of PERV-A (PERV-A 14/220/LacZ) to provide an independent confirmation that the cells were permissive for PERV-A infection. One clone obtained from this second round of screening was found to be reproducibly infectable. The library-encoded cDNA present in this clone was amplified by PCR and sequenced. Nucleotide-database analysis indicated that this molecule, which we call HuPAR-1, is nearly identical to GenBank accession no. FLJ11856 expressed from locus 75981 on chromosome 8. No known function has been reported for this molecule, although a nonintron ORF encompassing exon 3, but not the spliced cDNA form, has been identified as a putative G protein-coupled receptor, the ligand of which has yet to be defined (14). It is possible that the PERV-A receptor, similar to the chemokine receptors of HIV, is coupled to G proteins.

We identified in the GenBank database a closely related human gene to HuPAR-1 (HuPAR-2, GenBank accession no. FLJ10060, locus 55065, chromosome 17) as well as a murine homolog that is syntenic to FLJ10060 (GenBank accession no. AK008081, chromosome 5), both of unknown function. We cloned HuPAR-2 and AK008081 from the cDNA of human 293 cells and mouse testes, respectively. Alignment of the nucleotide sequences of HuPAR-1, HuPAR-2, and AK008081 identified regions of sequence conservation, which we used to isolate receptor homologs by RT-PCR from baboon testes, and from the porcine testes cell line ST-IOWA. We term these molecules BaPAR-2 (baboon) and PoPAR (porcine). We termed the baboon receptor homolog BaPAR-2, because the amino acid sequence has greater sequence similarity to HuPAR-2 (95.8%) than HuPAR-1 (85.7%) (Fig. 1*B*).

A comparison of the human PERV-A receptors and related homologs illustrated that the molecules are closely related (Fig. 1). Their hydrophobicity profiles indicated that they are multiple transmembrane-spanning proteins similar to other gammaretrovirus receptors (15), with 10 or possibly 11 transmembrane regions (Fig. 1*A*). Comparison of the amino acid sequence of these molecules indicated that HuPAR-2 and BaPAR-2 are related most closely, and that the porcine receptor and murine homologue are similarly distant from HuPAR-2/BaPAR-2 and HuPAR-1 (Fig. 1*B*).

Receptor Tropism and Localization Studies. When expressed in SIRC cells or the mouse NIH 3T3 cell line HuPAR-1, HuPAR-2, BaPAR-2, and PoPAR mediated both entry (Table 2) and productive replication of PERV-A (Fig. 2*A*). The prototype PERV-A LacZ (TELCeB-derived virus) showed a decreased titer for some human cells and SIRC/HuPAR-1 cells. It is possible that HuPAR-1 is less sensitive to certain PERV-A isolates than HuPAR-2. In contrast, expression of these receptors did not alter sensitivity of SIRC cells to PERV-B or -C (Table 2), indicating that these

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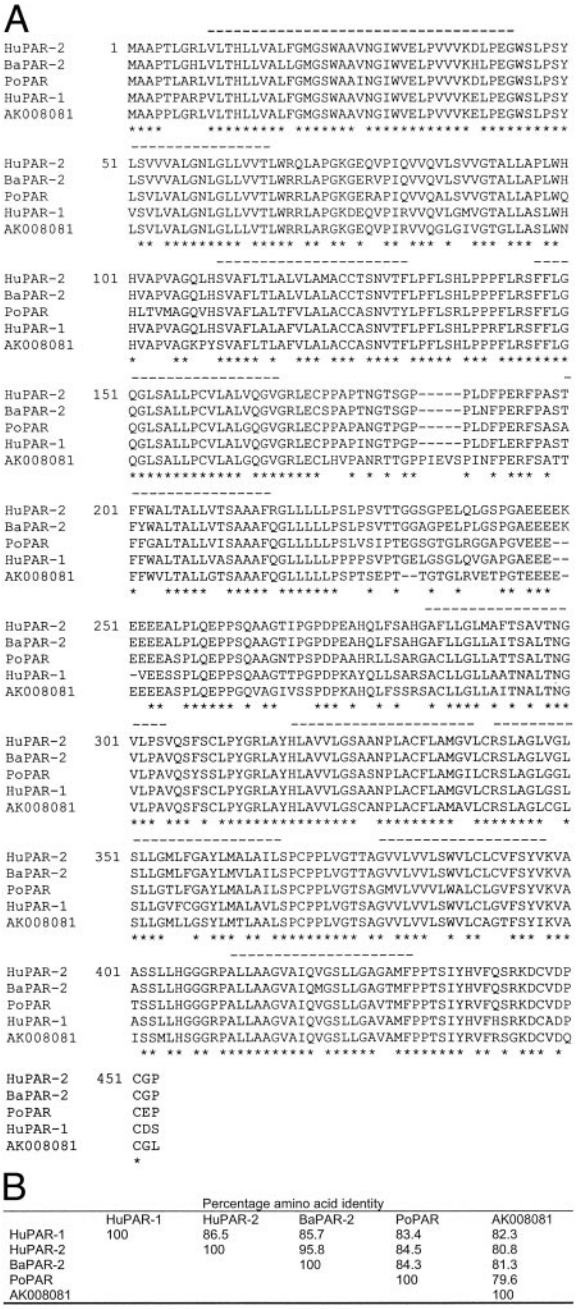

Fig. 1. Sequence comparison of the PERV-A receptors. (*A*) Amino acid alignment of the PERV-A receptors and murine homolog. Transmembrane domains predicted by TMPRED based on statistical analysis of TMbase (www.ch.embnet.org/software/TMPRED_form.html) are highlighted by overscores. Ten transmembrane domains were predicted in this model, whereas an alternative model using TMbase had 11 including the 10 domains shown. The amino acid residue number of the consensus sequence is indicated. (*B*) Amino acid sequence identity of the PERV-A receptors and the murine homolog AK008081.

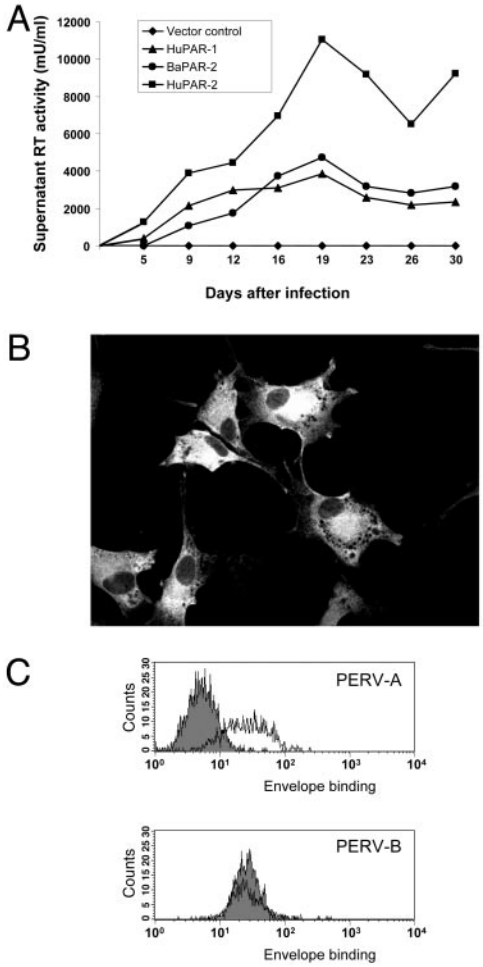

Fig. 2. Analysis of PERV receptor activity *in vitro*. (*A*) SIRC cells expressing HuPAR-1, HuPAR-2, or BaPAR-2 support productive replication of PERV. Cells were exposed to a PERV-A 14/220, and virus replication was assessed by measuring RT activity in the culture supernatants. Similar results were obtained with NIH 3T3 cells as well as for SIRC/PoPAR cells in separate experiments (results not shown). (*B*) HuPAR-2/EGFP protein is expressed at the plasma membrane of transduced SIRC cells. Intracellular protein, particularly in the perinuclear endoplasmic reticulum region, is also evident. (*C*) PERV-A but not PERV-B envelope binding is enhanced on SIRC cells by the expression of HuPAR-2 proteins. Envelope binding for parental SIRC (gray-filled histogram) and SIRC/HuPAR-2 (line without fill) cells. Specific binding of PERV-A Env to SIRC/PoPAR was observed (data not shown).

molecules do not function as receptors for either of these viruses. This specificity of function is consistent with the published observations that PERV-A, -B, and -C each belong to a distinct receptor interference group (5). Expression of the murine homologue (AK008081) in SIRC or NIH 3T3 cells did not mediate PERV-A infection (Table 2) as determined by the LacZ pseudotyping assays, consistent with our previous report that mouse cells lack a functional PERV-A receptor (5).

We investigated the cellular localization of HuPAR-2 proteins in SIRC cells by constructing a chimeric receptor tagged at the C terminus with EGFP. By using confocal microscopy, EGFP-tagged HuPAR-2 molecules were expressed at the plasma membrane as well as in the cytoplasm, particularly in the perinuclear region (Fig. 2*B*). Confocal analysis by z-series examinations in 0.2-μm steps confirmed the surface localization of the tagged protein (data not shown). LacZ pseudotype and TaqMan PCR infectivity assays on cells expressing the tagged HuPAR-2 protein excluded the possibility that the addition of the C-terminal EGFP had affected its ability to act as a receptor. In addition, this HuPAR-2/EGFP chimera supported infection by PERV-A as did an N-terminally tagged HuPAR-1 protein (Table 3). Interestingly, the indication in Table 2 and Fig. 2 that HuPAR-2 may mediate a higher level of PERV infection than HuPAR-1 is also seen in these tagged-receptor experiments by using different receptor constructs and assay systems.

We also used flow cytometry with His-tagged PERV-A envelope constructs to examine the binding of PERV-A envelope proteins to

Table 3. Tagged HuPAR proteins are functional for PERV-A infection

Cell line	LacZ pseudotype assay	Quantitative PCR
SIRC	<4	1.3×10^2
SIRC HuPAR-1/His	NT	2.5×10^5
SIRC HuPAR-2/EGFP	2×10^4	1.0×10^6
293	8×10^4	8.2×10^6
293 (no PERV)	<4	3.7×10^2
SIRC (no PERV)	<4	UD

The HuPAR-1/HIS and HuPAR-2/EGFP chimeric constructs possess N-terminal 6×-His and C-terminal EGFP tags, respectively. PERV-A infection was measured by plating of PERV-A 14/220/LacZ pseudotype virus and by quantitative real-time PCR, with PERV DNA copy numbers being determined from standard curves (correlation coefficient, 0.998). These data are representative of the results of three similar experiments. UD, undetectable (i.e., copy number values were below the confidence limits of the standard curve); NT, not tested.

cells expressing the receptors. The receptor-binding region of the PERV-A Env efficiently bound to SIRC cells expressing HuPAR-2 (Fig. 2*C*). This binding was specific to HuPAR-2 because (*i*) parental SIRC cells that do not express HuPAR-2 did not bind the PERV-A envelope, and (*ii*) binding of a PERV-B envelope construct was not enhanced by the expression of HuPAR-2 (Fig. 2*C*). Taken together, these results indicate that HuPAR-2 plays a role in binding PERV-A that is analogous to the known multitransmembrane receptors for other gammaretroviruses (15–17).

PERV-A Receptor Expression *in Vivo*. The study of the sensitivity of various human tissues to PERV infection studied in conjunction with PERV receptor expression may provide important information on xenotransplantation safety. PBMCs are the most accessible tissue and have been tested for the presence of PERV in patients exposed to live porcine tissues (18–22). However, sensitivity of PBMCs to PERV entry or infection has not been demonstrated unequivocally (3, 23). We developed an RT-PCR assay that differentiated between HuPAR-1 and HuPAR-2. Using this assay we detected expression of both HuPAR-1 and HuPAR-2 in PBMCs of 11 healthy volunteers (Fig. 3*A*). We found that human PBMCs are permissive to PERV-A infection (Fig. 3*B*). Furthermore we detected expression of PERV-A Gag proteins after PERV-A 14/220 infection of PBMC cultures using an immunostaining method (24).

Receptor expression was also detected in a wide variety of human tissues, with the possible exception of the bladder, by a Northern blot survey with a probe that detects both HuPAR-1 and HuPAR-2 (Fig. 3*C*). It is interesting that expression of the human PERV-A receptor was high in the testes. Although our investigations do not define which cell types within the tissue are expressing the receptors, it is possible that the high expression may be in the germ-line cells or cells comprising their developmental environment.

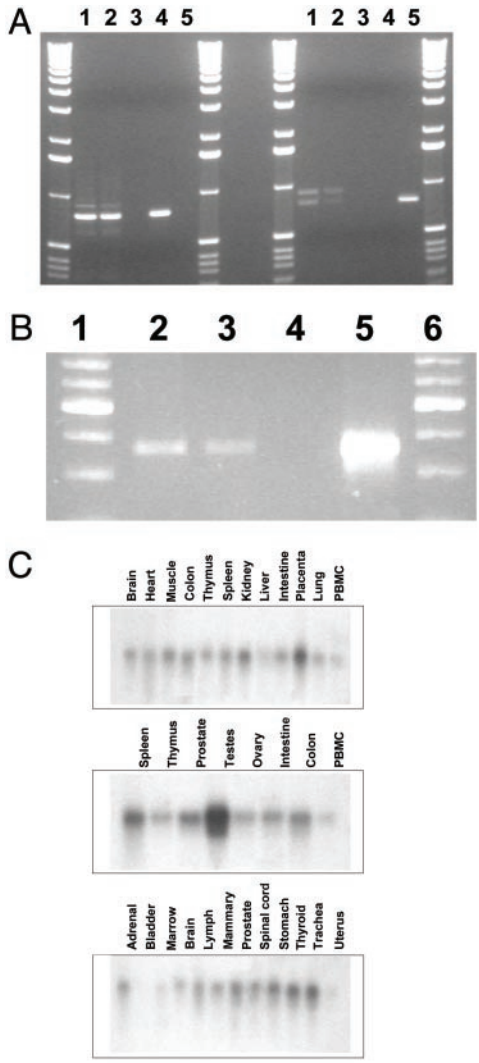

Fig. 3. *In vivo* expression and cellular localization of PERV-A receptors. (*A*) PBMCs express HuPAR-1 and HuPAR-2 RNA. RT-PCR products from total cellular RNA were electrophoresed. Lanes 1 and 2, human PBMCs; lane 3, SIRC control; lane 4, SIRC HuPAR-1; lane 5, SIRC HuPAR-2. Results from two typical volunteers are presented. All 11 volunteers expressed both HuPAR-1 and HuPAR-2 RNA. The presence of two products in the HuPAR-2 RT-PCR is due to the presence of a nonspecific amplicon. (*B*) Infection of primary human PBMCs by PERV-A 14/220. After exposure to PERV-A 14/220, PERV-A sequences were detectable by PCR for *pol* (data not shown) and *env* (shown) sequences. Lanes 1 and 6, marker; lanes 2 and 3, PBMCs on days 8 and 14 postinfection, respectively; lane 4, uninfected PBMCs; lane 5, 293 cells chronically infected with PERV-A 14/220. (*C*) Expression of PERV-A receptor RNA was determined in multiple human primary tissue samples by Northern blot with a probe that detects both HuPAR-1 and HuPAR-2.

Discussion

We have identified two functional human receptors for PERV-A. The use of multiple independent multitransmembrane receptors by a single retrovirus has been described for other gammaretroviruses such as baboon endogenous virus and some strains of MLV (10A1 strain) as well as feline leukemia virus (FeLV-B strain) variants (15). We show evidence for the expression of the receptor molecules at cellular membranes and specific binding of PERV-A Env to cells expressing the HuPAR-A2 receptor. These data are consistent with the well established concept that multitransmembrane gammaretrovirus receptors affect both Env binding and virus fusion (15, 25). It is noteworthy that previously characterized gammaretrovirus receptors serve as phosphate and amino acid transporters (15). These receptors become down-regulated after virus infection and can cause harmful effects on the infected host cells. Our preliminary data (not shown) also suggest that HuPAR-2/EGFP expressed on SIRC is down-regulated at the surface within 2 h of exposure to PERV infectious supernatant. However, without knowing the physiological role of the PERV-A receptors, it is impossible to predict the pathogenic consequences of PERV infection. In this regard, it is noteworthy that pigs express a functional PERV-A receptor and have carried endogenous PERV-A genomes for many millions of years (26), and PERV-A loci capable of infecting pig cells have been identified in some pig genomes (27).

Pathogenic consequences of human viral infections can often be revealed by the use of appropriate animal models. The use of nonhuman primates for PERV pathogenicity has been investigated (28, 29) because they have been used as models for clinical

xenotransplantation. We found that baboon cells express at least one functional receptor for PERV-A (Table 2 and Fig. 2), although the LacZ titer and RT assays suggest that it may not be as efficient as the most closely related human receptor (HuPAR-2). Until reagents are developed that can quantitate the cell-surface expression levels and therefore normalize these infectious assays by expression, it will not be possible to definitively test this observation. Therefore, although our results support the use of nonhuman primates, at least baboons, as a model to study PERV transmission *in vivo*, the question of whether the baboon receptor and thus the risk of PERV infection is the same as for humans remains. In this regard, it is noteworthy that not all primate, nor human, cells are permissive to PERV-A entry despite widespread expression of the PERV-A receptors (3, 5, 30). The identification of the PERV-A receptor will facilitate the development of reagents such as blocking antibodies to investigate whether these differences are related to receptor expression, posttranslational modifications, or cell-specific mechanisms regulating retroviral–cell interactions. Thus the identification of PERV-A receptors will facilitate dissection of the infection process and the possible identification of cellular mechanisms that enhance or prevent PERV infection.

We did not detect any receptor activity associated with the murine homolog of the PERV-A receptors with LacZ pseudotype assays, although more sensitive quantitative PCR studies indicate that murine cells may be nonproductively infected at low levels (data not shown). Previous reports have demonstrated that immunodeficient mice transplanted with pig islets can be infected by PERV but without evidence of productive infection (31, 32). It remains to be determined whether this infection represents infection of the mouse cells with PERV-A or whether it is the result of limited replication of PERV-B or -C. Although a functional PERV-B receptor has been demonstrated on murine cells (5), the isolation of replication-competent PERV-B from transmission assays with primary pig cells such as the pig islets used in one of the studies has not been reported (6, 7, 30).

We show that HuPAR expression is ubiquitous in most human tissues (Fig. 3), suggesting that PERV-A should have the ability to infect many tissue compartments. Functional receptor expression in PBMCs as well as their susceptibility to PERV infection supports the relevance of conclusions drawn from clinical studies that there was no evidence of PERV transmission in the PBMCs of patients exposed to pig tissues or cells (18–20, 22). However, *in vitro* studies have shown that few human and nonhuman primate cells are permissive for productive infection by PERV-A even if they are susceptible to PERV-A pseudotype entry (3, 5, 30, 33). Therefore, our results suggest that specific blocks to PERV replication both at the virus entry and postentry steps may exist. We recently published evidence that similar entry and postentry blocks for PERV infection exist for nonhuman primate cells, another issue for caution in considering the potential value of published experience with nonhuman primate models of PERV infection at this time (3, 5, 30, 33). The significance of such blocks on the *in vivo* tropism of PERV-A, and therefore ultimately the safety of clinical xenotransplantation, remains to be determined. Further *in vitro* and *in vivo* infection studies with high-titer PERV such as PERV-A 14/220 used in this study are required to address this possibility.

In this study we identify two closely related human molecules that are used by PERV-A to infect human cells. We further found that homologous genes from the baboon and the pig also encoded functional receptors. We speculate that HuPAR-1 and -2 may have duplicated after the divergence of rodents and pigs from primates but before the divergence of humans and baboons. It is possible, therefore, that baboons have an additional HuPAR homologue that may also function as a PERV-A receptor. In addition to humans, baboons, and pigs, the cells of some other species such as mink, cat, and dog have been shown to express functional receptors for PERV-A (5, 30). It remains to be determined how many genes homologous to HuPAR these species possess and how many of them are functional as PERV-A receptors. The functional porcine homolog will be interesting for the study of germ-line fixation and possible pathogenicity of PERV-A in pigs.

Small-animal models of PERV infection will be useful to explore factors such as immunosuppression that might affect susceptibility to infection *in vivo* as well as possible pathogenic consequences of infection. Furthermore, it should be possible to investigate the potential for PERV to be transmitted between animals. When the public health risks associated with xenotransplantation are considered, the potential transmissions of PERV from xenograft recipient to intimate contacts as well as the public at large is a major concern. Thus, although a properly informed individual patient may choose to accept the risks of PERV transmission even with the risk of a hematological malignancy or other disease, they cannot consent to the risks to their contacts or the public. Our results show that the block to PERV-A replication in murine cells is solely at the receptor level, because the transfer of the receptor gene into murine cells rendered them permissive to PERV-A replication. Therefore a small-animal model should be achievable by the production of PERV-A receptor transgenic mice.

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1. Chapman, L. E. & Bloom, E. T. (2001) *J. Am. Med. Assoc.* **285,** 2304–2306.
2. Takeuchi, Y. & Weiss, R. A. (2000) *Curr. Opin. Immunol.* **12,** 504–507.
3. Patience, C., Takeuchi, Y. & Weiss, R. A. (1997) *Nat. Med.* **3,** 282–286.
4. Le Tissier, P., Stoye, J. P., Takeuchi, Y., Patience, C. & Weiss, R. A. (1997) *Nature* **389,** 681–682.
5. Takeuchi, Y., Patience, C., Magre, S., Weiss, R. A., Banerjee, P. T., Le Tissier, P. & Stoye, J. P. (1998) *J. Virol.* **72,** 9986–9991.
6. Wilson, C. A., Wong, S., Muller, J., Davidson, C. E., Rose, T. M. & Burd, P. (1998) *J. Virol.* **72,** 3082–3087.
7. Oldmixon, B. A., Wood, J. C., Ericsson, T. A., Wilson, C. A., White-Scharf, M. E., Andersson, G., Greenstein, J. L., Schuurman, H. J. & Patience, C. (2002) *J. Virol.* **76,** 3045–3048.
8. Coffin, J., Hughes, S. & Varmus, H. (1997) *Retroviruses* (Cold Spring Harbor Lab. Press, Plainview, NY).
9. Wong-Staal, F., Reitz, M. S., Jr., & Gallo, R. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2032–2036.
10. Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A. & Collins, M. K. (1995) *J. Virol.* **69,** 7430–7436.
11. Miller, A. D. & Rosman, G. J. (1989) *Biotechniques* **7,** 980–990.
12. Chen, C. A. & Okayama, H. (1988) *Biotechniques* **6,** 632–638.
13. Sugai, J., Eiden, M., Anderson, M. M., Van Hoeven, N., Meiering, C. D. & Overbaugh, J. (2001) *J. Virol.* **75,** 6841–6849.
14. Takeda, S., Kadowaki, S., Haga, T., Takaesu, H. & Mitaku, S. (2002) *FEBS Lett.* **520,** 97–101.
15. Overbaugh, J., Miller, A. D. & Eiden, M. V. (2001) *Microbiol. Mol. Biol. Rev.* **65,** 371–389.
16. Farrell, K. B., Ting, Y. T. & Eiden, M. V. (2002) *J. Virol.* **76,** 4267–4274.
17. Farrell, K. B., Russ, J. L., Murthy, R. K. & Eiden, M. V. (2002) *J. Virol.* **76,** 7683–7693.
18. Paradis, K., Langford, G., Long, Z., Heneine, W., Sandstrom, P., Switzer, W. M., Chapman, L. E., Lockey, C., Onions, D. & Otto, E. (1999) *Science* **285,** 1236–1241.
19. Elliott, R. B., Escobar, L., Garkavenko, O., Croxson, M. C., Schroeder, B. A., McGregor, M., Ferguson, G., Beckman, N. & Ferguson, S. (2000) *Cell Transplant.* **9,** 895–901.
20. Dinsmore, J. H., Manhart, C., Raineri, R., Jacoby, D. B. & Moore, A. (2000) *Transplantation* **70,** 1382–1389.
21. Heneine, W., Switzer, W. M., Soucie, J. M., Evatt, B. L., Shanmugam, V., Rosales, G. V., Matthews, A., Sandstrom, P. & Folks, T. M. (2001) *J. Infect. Dis.* **183,** 648–652.
22. Heneine, W., Tibell, A., Switzer, W. M., Sandstrom, P., Rosales, G. V., Mathews, A., Korsgren, O., Chapman, L. E., Folks, T. M. & Groth, C. G. (1998) *Lancet* **352,** 695–699.
23. Martin, U., Winkler, M. E., Id, M., Radeke, H., Arseniev, L., Takeuchi, Y., Simon, A. R., Patience, C., Haverich, A. & Steinhoff, G. (2000) *Xenotransplantation* **7,** 138–142.
24. Bartosch, B., Weiss, R. A. & Takeuchi, Y. (2002) *J. Gen. Virol.* **83,** 2231–2240.
25. Hunter, E. (1997) in *Retroviruses*, eds. Coffin, J. M., Hughes, S. H. & Varmus, H. E. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 71–120.
26. Patience, C., Switzer, W. M., Takeuchi, Y., Griffiths, D. J., Goward, M. E., Heneine, W., Stoye, J. P. & Weiss, R. A. (2001) *J. Virol.* **75,** 2771–2775.
27. Niebert, M., Rogel-Gaillard, C., Chardon, P. & Tonjes, R. R. (2002) *J. Virol.* **76,** 2714–2720.
28. Blusch, J. H., Patience, C., Takeuchi, Y., Templin, C., Roos, C., Von Der Helm, K., Steinhoff, G. & Martin, U. (2000) *J. Virol.* **74,** 7687–7690.
29. Specke, V., Tacke, S. J., Boller, K., Schwendemann, J. & Denner, J. (2001) *J. Gen. Virol.* **82,** 837–844.
30. Wilson, C. A., Wong, S., VanBrocklin, M. & Federspiel, M. J. (2000) *J. Virol.* **74,** 49–56.
31. van der Laan, L. J., Lockey, C., Griffeth, B. C., Frasier, F. S., Wilson, C. A., Onions, D. E., Hering, B. J., Long, Z., Otto, E., Torbett, B. E. & Salomon, D. R. (2000) *Nature* **407,** 90–94.
32. Deng, Y. M., Tuch, B. E. & Rawlinson, W. D. (2000) *Transplantation* **70,** 1010–1016.
33. Ritzhaupt, A., Van Der Laan, L. J., Salomon, D. R. & Wilson, C. A. (2002) *J. Virol.* **76,** 11312–11320.