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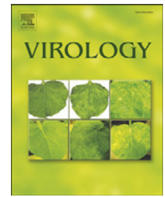

Host range and cellular tropism of the human exogenous gammaretrovirus XMRV

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ABSTRACT

Recently, the first human infection with an exogenous gammaretrovirus (XMRV) was reported. In its initial description, XMRV was confined to prostate stromal fibroblasts, although subsequent reports demonstrated XMRV protein expression in prostate epithelial cells. Most recently, XMRV has been detected in blood cells of patients with chronic fatigue syndrome. The aim of this study was to elucidate the transmission routes and tissue tropism of XMRV by comparing its host range, receptor usage and LTR functionality with other MLV isolates. We demonstrate using pseudotype experiments that XMRV Env mediates efficient infection of cells from different species. We show that replication competent XMRV infects various human cell types, including hematopoietic cell lines and prostate stromal fibroblasts. XMRV–LTR activity is significantly higher in the prostate cancer cell line LNCaP and in prostate stromal fibroblasts, compared to other cell types tested and could be one factor contributing to efficient viral spread in prostate tissue.
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Introduction

Recently, a new gammaretrovirus associated with a rare form of familial human prostate cancer, which was named XMRV due to its homology to xenotropic murine leukemia viruses (X-MLV), was discovered (Urisman et al., 2006). The virus was detected almost exclusively in samples isolated from patients that are homozygous for a specific *RNASEL* allele, demonstrating a strong link between XMRV infectivity and reduced RNase L activity. A second study in North America confirmed a high incidence of XMRV in prostrate cancer samples, which was independent of the RNASEL allele (Schlaberg et al., 2009). In studies conducted in Northern Europe, however, the virus was rarely detected in unselected prostate cancer samples, thus suggesting differences in the worldwide distribution of XMRV (Fischer et al., 2008; Hohn et al., 2009). Two recent studies have provided evidence with regard to the putative transmission routes of XMRV; Hong and colleagues propose that XMRV might be sexually transmitted (Hong et al., 2009) and a recent finding by Lombardi and coworkers raises the possibility of XMRV being a blood borne pathogen (Lombardi et al., 2009). In this latter study, XMRV-specific sequences were detected in up to 70% of peripheral blood monocytes (PBMCs) from patients with chronic fatigue syndrome (CFS).

Immunohistochemistry studies have confirmed the presence of XMRV in prostrate samples, but FISH-positive staining for XMRV was restricted to only a very few fibroblasts (~1%) of the prostate stroma (Urisman et al., 2006), indicating a tight restriction of virus spread in vivo. In a more recent study in which XMRV-specific antiserum was used, XMRV protein expression was detected in epithelial cells of unselected prostate cancer tissues (Schlaberg et al., 2009), suggesting a more direct involvement of XMRV in prostate cancer tumorigenesis. Together with the finding that XMRV sequences are also detected in the prostate epithelium cell line 22Rv1 (Knouf et al., 2009), these studies indicate that XMRV infection might not be limited to prostate stromal fibroblasts in vivo.

The XMRV provirus is not endogenous to the human genome and there are no similarities to human endogenous retroviral sequences, suggesting that XMRV is transmitted by exogenous infection. Due to high nucleotide identity (95%) with several full-length *Mus musculus* endogenous and exogenous proviruses, it is likely that the mouse is the original source of the virus–but it is probably not the current reservoir for infections. As XMRV is the first example of an extant human exogenous gammaretrovirus, its existence raises many questions with regard to the etiological link between retroviral infection and cancer, but also to the nature of the virus reservoir, route of transmission and the integrity of cellular barriers that have evolved to control retroviral infections. To address the latter, it is necessary to understand the cellular and retroviral factors that modulate XMRV infection.

An important modulator of virus host range and tissue specificity is the interaction of the cellular receptor and the retroviral Env. Based on

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the fact that XMRV shares the highest amino acid identity within the SU domain (87% for VRA and 78% for VRB) with different xenotropic MLVs (e.g., NZB-9-1, NFS-Th-1 und DG75) (Urisman et al., 2006), the cellular receptor for XMRV was predicted to be XPR1, a multi-membrane-spanning molecule identified as the receptor for xenotropic and polytropic MLVs (Battini et al., 1999; Tailor et al., 1999; Yang et al., 1999). This prediction was confirmed by overexpression of the human XPR1 receptor protein in non-permissive NIH3T3 and CHO cells which renders them permissive for XMRV infection (Dong et al., 2007). However, studies have not been performed to compare the infection efficiencies of different cell types when mediated by either xenotropic or XMRV env proteins. Furthermore, although RNA analyses has indicated a ubiquitous expression of XPR1 in human tissue (Battini et al., 1999; Tailor et al., 1999), the localization of XPR1 within the plasma membrane of different cell types has not been previously addressed.

In addition to the envelope protein, the long terminal repeat (LTR) region is the second major determinant of retroviral tropism. Enhancer elements that specifically interact with transcription factors are localized within the promoter region, in particular within the U3 region. The LTR of XMRV is 535 nucleotides long and has the highest nucleotide identity (96%) with LTRs from xenotropic MLVs (Fig. 5A) (Urisman et al., 2006). However, single nucleotide substitutions and an insertion of AG dinucleotide immediately downstream from the TATA box have been described for XMRV (Urisman et al., 2006).

This study was initiated to determine whether the XMRV tissue distribution in vivo was related to cell type-specific receptor expression and/or usage, or due to the expression of cellular factors essential for LTR activity. Our study documents that XMRV restriction in vivo can only be partially explained by the two major determinants of retroviral tropism: receptor distribution and LTR function. XMRV env pseudotyped particles, as well as replication competent XMRV, show a typical xenotropic host range infecting feral mouse cells, all types of human cells tested and several non-rodent species. XMRV binding experiments indicate that although XPR1 is ubiquitously expressed in all cell types analyzed, its functional localization on the plasma membrane varies between different cell types. Furthermore, XMRV–LTR activity is significantly higher in primary stromal fibroblasts isolated from prostate tissue or the prostate cancer cell line LNCaP, as compared to other cell types. The increased promoter activity in the prostate compartment was also observed for related MLV LTRs and is therefore not unique to XMRV LTR.

Results

Analysis of the cell type specificity of the XMRV Env pseudotypes

The restricted infection pattern of XMRV in stromal fibroblast observed in patient samples in vivo is in striking contrast to earlier in vitro data demonstrating that a wide range of cell types are infectable by X-MLVs. To test the host range of XMRV, cell lines of different tissue origins were tested for infection with XMRV Env pseudotyped MLV particles containing a retroviral vector expressing GFP. As controls, infection efficiencies of Env-pseudotypes representing different gammaretrovirus receptor groups were compared. These include the xenotropic NZB-MLV, which uses XPR1 as a receptor and the recombinant 10A1-MLV, which can use both phosphate transporter proteins PiT1 and PiT2 as putative receptor molecules (Battini et al., 1999; Miller and Chen, 1996). Additionally, ecotropic env pseudotyped virus particles (using the amino acid transporter CAT-1 on mouse cells) were included as negative controls (Table 1).

Cell type specificity was determined on a number of human cell lines, including the LNCaP and DU145 prostate cancer cells, primary cells from human umbilical vein endothelial cells (HUVEC) and primary prostate stromal fibroblasts (PrSc) (Table 1). The PrSc cells were of particular interest due to the restricted infection pattern of XMRV observed in vivo. Strikingly, XMRV efficiently infects all human cells lines and primary cells tested. Infection titers of XMRV Env pseudotypes on stromal cells, whose identity was confirmed by immunohistochemical staining (cytokeratin negative and vimentin positive; data not shown), were indistinguishable from other human cell lines, demonstrating that PrSc cells are not preferentially targeted by XMRV Env–receptor interactions as measured ex vivo.

Although, all the different human cell types used here were receptive to both XMRV and X-MLV pseudotype infections, relative infection efficiencies varied between cell lines, but also between virus types (Table 1). For instance, Jijoye and REH B-cells and primary HUVEC endothelial cells were more efficiently infected with XMRV Env pseudotypes, with approximately a five-fold higher titer than observed with X-MLV pseudotypes (Table 1, boxed), whereas a two-fold lower titer was observed for XMRV on LNCaP and PrSc cells (Table 1).

We next asked if the XMRV Env can also mediate infection of cell lines from other animal types (Fig. 1). For this study, a wide spectrum of cells from different species (human, hamster, mink, pig, cow and monkey) was subjected to Env pseudotyped infection assays.

Table 1
Host range analysis of pseudotyped murine leukemia viruses.

Cell line[a]	Titers (GFP iU × 10^5/ml) of Env pseudotyped MLV[b]			
	XMRV	Xeno	10A1	Eco
TE 671	9.5	13	6.2	nd[c]
HeLa	5.6	4.8	4.0	nd[c]
Jijoye	0.4	0.09	0.4	nd[c]
DU145	2.9	3.0	1.8	nd[c]
LNCaP	1.2	3.0	2.2	nd[c]
293T	2.4	2.8	0.95	nd[c]
REH	0.08	0.02	0.08	nd[c]
HL60	0.5	0.25	0.65	nd[c]
PrSc	1.9	4.2	0.3	nd[c]
HUVECs	3.0	0.41	0.27	nd[c]
NIH3T3	nd	nd	2.3	6.3

[a] Cell lines used in infection experiments are of human origin with exception of the mouse cell line NIH3T3.
[b] Cells were infected with Env pseudotyped SF91-GFP retroviral vector with an MOI of 4. GFP iU/ml was determined by FACS analyses. Titers were calculated as the arithmetic mean of three independent experiments. Boxed values label statistically significant differences in titers between the two xenotropic pseudotyped viruses. PrSc stands for prostatic stromal cells.
[c] nd, not detectable.

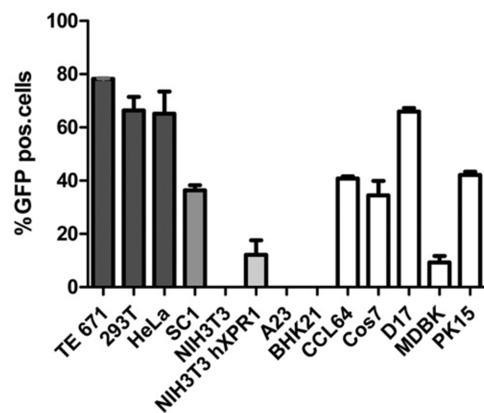

Fig. 1. XMRV env pseudotyped particles display xenotropic host range. Human cell lines (dark gray bars), feral mouse cell line SC1 (gray bar), inbred mouse cells NIH3T3, hamster cells A23 and BHK, mink cells CCL64, monkey cells Cos7, doc cell line D17, cells of bovine origin (MDBK) and porcine cells PK15 were infected with XMRV Env pseudotyped SF91-GFP retroviral supernatant (MOI 8). GFP-positive cells were measured 72 h after transfection using FACS analysis. Cell lines from species other than human and mouse are illustrated as white bars. The experiment was replicated four times using triplicates.

Consistent with the xenotropic interference group, pig PK15 cells, dog D17 cells, monkey COS7 and mink CCL64 cells were highly permissive for virus infection. Bovine MDBK cells were also susceptible to XMRV Env pseudotyped particles, although to a lesser extent, whereas hamster cell lines BHK21 and A23 were restricted to XMRV Env infection. In general, we found that all human cell lines tested were susceptible to XMRV Env infections, yielding higher infectious titer units as compared to cell lines from other species.

Transient expression of hXPR1 conferred infectivity to the non-permissive inbred mouse cell line NIH3T3 cells, although to lesser extent as compared to human cell lines (Fig. 1). Similar differences were observed when human XPR1 was transiently expressed in non-permissive CHO cells and A23 cells (data not shown), as well as in experiments using stable transfection of the XPR1 receptor in NIH3T3 cells (data not shown).

Infection and viral spread of replication competent XMRV

To test the ability of XMRV to productively infect human cells, a full-length proviral clone was constructed using two overlapping XMRV fragments from a patient VP62 to generate XMRV infectious particles (Dong et al., 2007; Urisman et al., 2006). After transfection of proviral DNA into various human cell lines, as well as primary stromal fibroblasts isolated from prostate tissue, supernatant was harvested at different time points and used to infect the indicator cell line LNCaP. Infection of LNCaP cells was monitored using a highly sensitive PCR method. Surprisingly, only in the case of 293T cells and the prostate cancer cell line LNCaP, which shows low RNase L activity, could production of XMRV be detected (Table 2). No virus release could be detected from TE671 cells, HeLa cells, the prostate cancer cell line DU145 or the human epithelial cell line A549.

Replication of XMRV in human cell lines was further investigated by infecting cells with XMRV containing supernatant from LNCaP cells chronically infected with XMRV. Infected cells were monitored for XMRV replication over a period of 21d by isolating RNA after 5d, 8d, 12d and 21d past infection followed by real-time RT-PCR specific for XMRV. Replication of XMRV could be observed in HeLa cells, the prostate cancer cell lines DU145 and LNCaP, epithelial lung cell line A549, TE671 cells, and primary stromal fibroblasts isolated from prostate tissue (Table 2). The levels of XMRV transcripts were consistently lower in 293T cells, suggesting less efficient XMRV replication in these cells. Despite stable detection of XMRV transcripts over the 21-day period in all cell lines, viral particles could only be detected in cell supernatants from LNCaP, PrSc, and 293T cells by Western blotting (Table 2).

XMRV infectious pseudotypes produced from chronically infected 293YFP cells (carrying a SF91-YFP retroviral vector) could efficiently infect various human cell lines, as well as primary cells, as determined by FACS analysis (Table 3). 293T and TE671 cells showed the highest levels of XMRV infectivity, while infection titers on HUVEC cells were reduced by a factor of five and by a factor of 10 on primary stromal fibroblast and human HL60 promyeloblasts. The observed reduction in viral infection titers is inline with reduced proliferation rates of PrSc, HUVEC's and HL60 cells compared to TE671 and 293T cells.

Table 2
XMRV producer cell lines and XMRV replication in human cell lines.

Cell line	RT-PCR from different cell lines	Viral particles release[a]	PCR from indicator cells[b]
TE 671	+	−	+
HeLa	+	−	+
293T	(+)	(+)	(+)
Du145	+	−	+
LNCaP	+	+	+
A549	+	−	+
PrSc	+	+	+

Cells were transfected with XMRV proviral DNA. Supernatant was collected every 24 h from the indicated cell lines for a time period of 21 days, filtered and subsequently applied to the indicator cell line LNCaP, which were then analyzed by nested PCR for the presence of viral DNA 72 h after infection. Viral particles (released from the cells transfected with proviral DNA) concentrated by ultracentrifugation were analyzed by Western blotting for the expression of gag-CA p30. + denotes release of viral particles measured by immunoblotting[a] or XMRV-specific sequences in the indicator cell line LNCaP[b].

XPR1 is an ubiquitously expressed plasma membrane protein

To determine whether differences in infection efficiencies reflected receptor expression levels, immunoblot analysis was performed on all cell lines tested. Strikingly, no strict correlation between XPR1 expression levels and infection efficiency of XMRV-pseudotypes was observed (Fig. 2 and Table 1). In a second approach to assess XPR1 expression levels, we used a chimeric antibody ($XMRV_{RBD}rFc$) encompassing the receptor binding domain (RBD) and the proline rich region (PRR) of XMRV Env, fused to the constant fragment of a rabbit immunoglobulin rFc. Earlier work has shown that expression of N-terminal truncated RBD in cells produce soluble proteins that fold autonomously, bind their receptor, and interfere with infection (Barnett et al., 2003; Battini et al., 1995; Battini et al., 1996; Fass et al., 1997; Heard and Danos, 1991; Kim et al., 2004; Lavanya et al., 2008). After confirming expression of $XMRV_{RBD}rFc$ protein by Western blot analysis (data not shown), the functionality was tested by infection–interference experiments (Fig. 3A). Infection of TE 671 and SC1 cells with XMRV Env pseudotyped particles was efficiently blocked (37% and 26% of initial levels, respectively) by the addition of $XMRV_{RBD}rFc$ containing supernatant (Fig. 3A). In contrast, only marginal reduction in infectivity was observed when culture medium from mock-transfected cells was used. Furthermore, addition of $XMRV_{RBD}rFc$ had only minor effects on 10A1 infection (80% residual infection in TE 671 cells and 85% in SC1 cells), demonstrating the specificity of inhibition of $XMRV_{RBD}rFc$ for XMRV infections.

To further verify that the XMRV-RBD domain actually recognized XPR1, culture medium containing $XMRV_{RBD}rFc$ was used in cell binding experiments with non-permissive and permissive cells, as well as originally non permissive NIH3T3 cells transiently expressing the human variant of Xpr1 (Figs. 3B and C). Binding of the XMRV Env-derived RBD to the target cell surface was quantified using an anti rabbit-FITC antibody and subsequent FACS analysis (Fig. 3C). This analysis gave consistent results to that observed by Western blot analysis (compare Fig. 2), but also confirmed that the receptor was located in the plasma membrane and thus assessable to XMRV binding. These studies thus show that $XMRV_{RBD}rFc$ protein can be used as an alternative to XPR1 antisera to study receptor localization and functionality. However, different binding efficiencies between $XMRV_{RBD}rFc$ and Xpr1 orthologues have not been analyzed and may account for observed variability between different species; a problem also inherent to different antisera.

Table 3
Infection of human cells with replication competent XMRV.

Replication competent virus	YFP iU × 10^4/ml				
	LNCaP	293T	PrSc	HUVECs	HL60 Z
XMRV	1.0	4.0	0.1	0.28	0.12

Cells were infected with an MOI of 4 (determined on TE671 cells) using supernatant from 293YFP cells transfected with a replication competent XMRV provirus and cultured for 2 months. YFP iU/ml was determined by FACS analyses. Titers were calculated as the arithmetic mean of three independent experiments performed in triplicates.

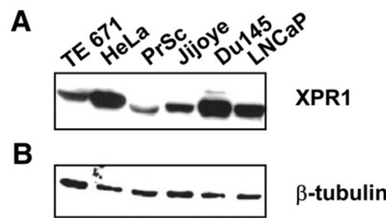

Fig. 2. XPR1 expression levels in cell lines. Immunoblot analysis of total protein extracts from various human cell lines. 25 μg of total protein per lane was separated on a 10% SDS gel, probed with anti-human XPR1 antibody (A) and reprobed to ensure equal protein amounts using an anti-β tubulin antibody (B).

XMRV–LTR activity is increased in cells from prostate compartment

Our experiments indicate that receptor distribution can not explain the observed in vivo restriction of XMRV infection. In addition, we found that only a few cell lines support efficient XMRV spread. Therefore, we sought to explore whether XMRV–LTR activity may be a factor for the observed results with regard to XMRV infection in vivo and in vitro. The LTR encompassing the complete U5-R-U3 region was fused to firefly luciferase gene (Fig. 4A), transiently transfected in different human and mouse cell lines, analyzed for transcription and normalized using a renilla luciferase expression construct (Fig. 4A). In each cell line, the relative activity of XMRV LTR was determined with respect to Herpes simplex virus TK promoter driven luciferase activity. 293T and HeLa cells were chosen because they generally support high levels of expression from a variety of different promoters. Additionally, prostrate cell lines, LNCaP and DU145 and primary stromal fibroblasts established from two different patients were analyzed for promoter activity. With the exception of 293T cells, all cell lines tested showed robust XMRV–LTR activity (Fig. 4B). Interestingly, transcriptional activity was the highest in cell lines that supported XMRV spreading: the prostate cancer cell line LNCaP and primary prostatic stromal fibroblasts. LTR activity was significantly reduced in HeLa cells, as well as in the prostate cancer cell line DU145. To our surprise, 293T cells which could be chronically infected with XMRV demonstrated only marginal XMRV–LTR transcriptional activity, but which may explain the relatively low levels of XMRV transcripts observed in these cells after infection.

Comparison of XMRV–LTR activity to other full-length retroviral LTRs

In addition to testing the XMRV LTR in the luciferase transcription assays, we also tested two full-length MLV LTRs that are distinct to XMRV and typically used as enhancer/promoter units in retroviral vectors, namely the LTRs from Moloney (Mo)-MLV, and Friend spleen focus forming virus (SFFV). A phylogenetic tree of the U3-R LTR region of all LTRs used in this study and, additionally, the xenotropic NZB-MLV LTR is shown in Fig. 5A. Both LTR transcription plasmids were compared to the promoter activity of XMRV LTR in 293T, HeLa, LNCaP and stromal fibroblasts (Fig. 5B). We did not see significant differences with regard to cell specific transcriptional activity of the LTR regions tested, with the exception of 293T cells. XMRV LTR is the only promoter in our experimental setting possessing only marginal transcription activity in 293T cells; MoMLV LTR and SFFV LTR displayed robust luciferase expression. Furthermore, we found that increased transcriptional activity in prostate cell line LNCaP and prostate stromal fibroblasts is not unique to XMRV LTR; all three LTR constructs possess increased transcriptional activity in prostate stromal fibroblasts as well as in the prostate cancer cell line LNCaP.

Discussion

Recently, the first human infection with a xenotropic murine leukaemia virus has been reported; XMRV was identified in tissue

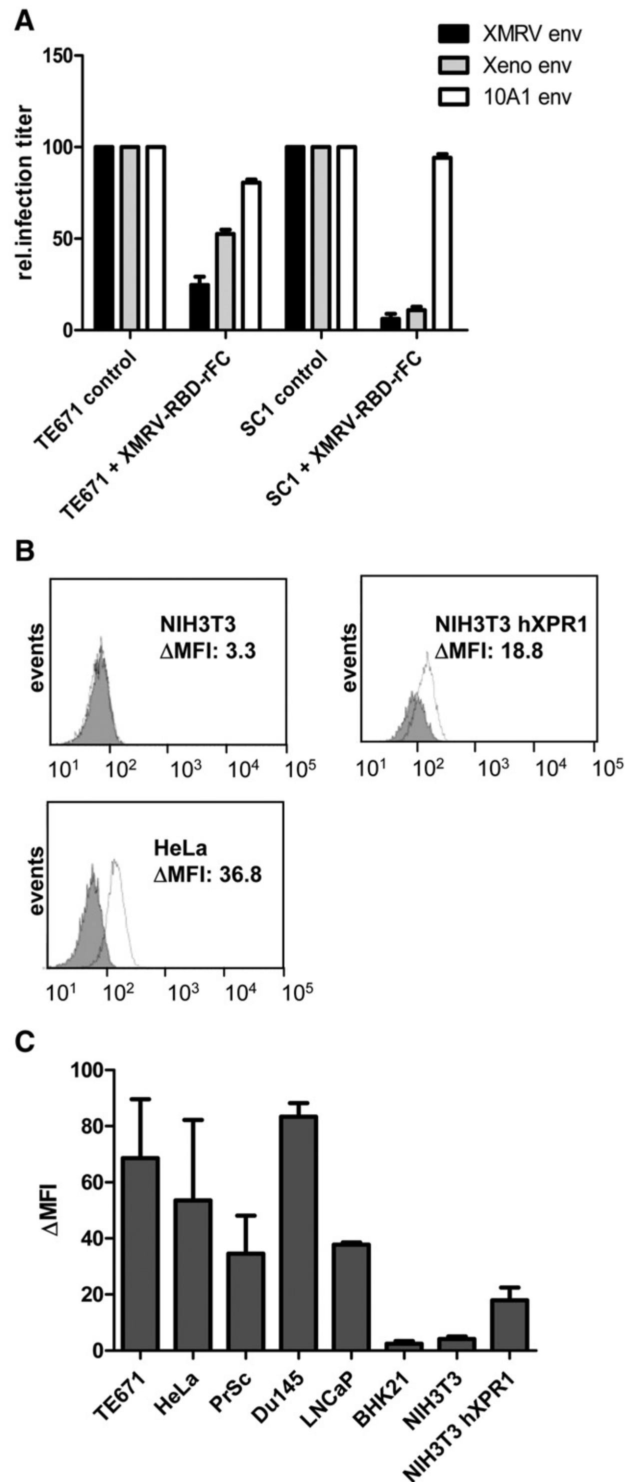

Fig. 3. (A) $XMRV_{RBD}$rFc efficiently blocks binding. TE 671 cells or feral mouse cells SC1 were preincubated with culture medium containing $XMRV_{RBD}$rFc or control medium and subsequently infected with XMRV env, Xeno env or 10A1 env pseudotyped MLVs. Infectivity was measured by FACS analysis 3 days after infection. Infection of cells is indicated as relative infection levels of one representative experiment out of four. (B) Env-binding assays using $XMRV_{RBD}$rFc fusion proteins. Binding assays were performed using XMRV permissive human cell line HeLa, the non-permissive mouse cell line NIH3T3 and NIH3T3 cells transiently transfected with the human XPR1 variant. Cells were incubated with culture medium from cells expressing $XMRV_{RBD}$rFc (empty histograms) or with culture medium from Mock transfected cells (gray-filled histograms) and analyzed by FACS after incubation with anti-rabbit FITC IgG. (C) Δ mean fluorescence intensity values (ΔMFI) were used to quantify binding of $XMRV_{RBD}$rFc to different human cell lines, mouse cell line NIH3T3 and the hamster cell line BHK21.

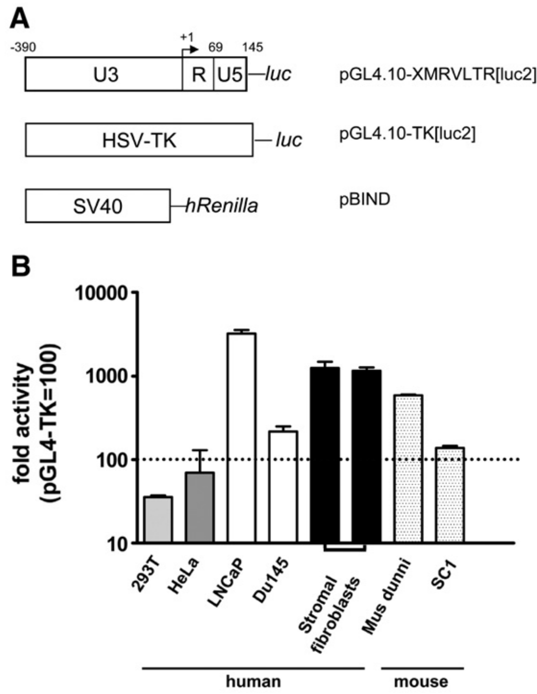

Fig. 4. (A) LTR-luc reporter construct and control reporter constructs. pGL4-TK was used as positive control and pBIND for normalization purposes. (B) XMRV–LTR activity in human and mouse cell lines. U3-R-U5 LTR luciferase activity in human cell lines 293T (light gray bar), HeLa (dark gray bar), the prostate cancer cell lines LNCaP and Du145 (white bars), in human primary stromal fibroblast isolated from two different patients (black bars) as well as in feral mouse cell line SC1 and in mus dunni cells (dotted bars) was determined 24 h after transfection. The experiment was four times repeated using triplicates.

from patients with familial prostate cancer mutated in *RNASE L* (Urisman et al., 2006). In addition to HIV and HTLV, which belong to the lenti- and δ-retrovirus genera, respectively, XMRV is the third class of exogenous infectious retroviruses found to replicate in humans. Two recent reports indicate that XMRV infection might have a larger impact on human diseases: XMRV protein expression was described predominantly in epithelial cells of the prostate in up to 25% of all prostate cancers analyzed (Schlaberg et al., 2009) and XMRV-specific sequences were identified in 67% of PBMCs of patients with CFS (Lombardi et al., 2009). These observations, which still need confirmation by other groups, evoke questions about the tissue tropism of the virus and the cellular factors that govern infection. Therefore, knowledge of the host range of XMRV and the cell types susceptible to infection is necessary to determine the potential reservoirs of the virus. In this study we characterized the Env–receptor interactions that dictate XMRV infection in vitro, providing information with regard to cell and tissue tropism of XMRV and its potential host range. Additionally, we present data regarding expression levels and plasma membrane localization of the XMRV receptor, XPR1. Furthermore, we demonstrate that XMRV promoter activity significantly varies in different cell lines, with the prostate cancer cell line LNCaP and primary prostate stromal fibroblasts yielding highest promoter activity.

By infecting cell lines from various species with XMRV Env pseudotyped particles, we showed that XMRV indeed possesses the expected "xenotropic" host range (Urisman et al., 2006). XMRV Env pseudotyped particles are unable to infect cells derived from laboratory mouse strains or from hamsters, but can infect cells from outbred feral mice, as well as mink, dog, pig cow and monkey–although with significant lower efficiency compared to human cells. Our results are supported by recently published data describing six distinct host range variants among naturally occurring xenotropic/polytropic MLV with XMRV and AKR6 MLV (xenotropic MLV) defining one distinct host range type (Yan et al., 2009). The efficient infection of human cells could be attributed to efficient binding to the human XPR1 variant, as shown by infection and binding assays using XMRV non-permissive cells NIH3T3, genetically engineered to express the human XPR1 variant. It is important to note that the ability of XMRV Env to direct infection to other animals means that the virus reservoir for XMRV infections may not be limited to mouse or man.

In light of the fact that XMRV uses XPR1 as a receptor and due to the broad tissue expression of XPR1 (Battini et al., 1999), it would be expected that XMRV would be able to infect a wide spectrum of tissues–in contrast to its limited cell-type distribution observed in prostrate tumors. Indeed, we could show that XMRV Env, like other xenotropic Env, is able to mediate infection of many human cell types, including lymphocytes, primary cells of epithelial origin and cells of prostatic stromal origin. Our observations are in concordance with data from Hong and colleagues who demonstrated that prostatic stromal cells, prostatic epithelial cells and cells from lymphoid origin can be efficiently infected with replication competent XMRV (Hong et al., 2009). Generally, there was no statistically significant difference between the infection efficiencies of XMRV and X-MLV Env pseudotyped particles on different human cell types, although XMRV infection frequencies were five-fold higher on B lymphocytes (Jijoye and REH) and HUVEC cells, but lower by a factor of two on primary prostate fibroblasts and established LNCaP cells. The reason for the observed difference is unclear, but they demonstrate that the XMRV Env is not functionally equivalent to other X-MLV.

Furthermore we show that the variations in virus titers of XMRV–Env pseudotypes on various human cells, with similar proliferation rates, could not be solely explained by differences in receptor expression. Using different techniques that detect XPR1 at either the protein level (Western blot analysis, immunofluorescence, FACS analysis) or RNA level (RT-PCR), a strict correlation between the amount of receptor being expressed and infectivity of the cells could not be observed, indicating that other cellular factors

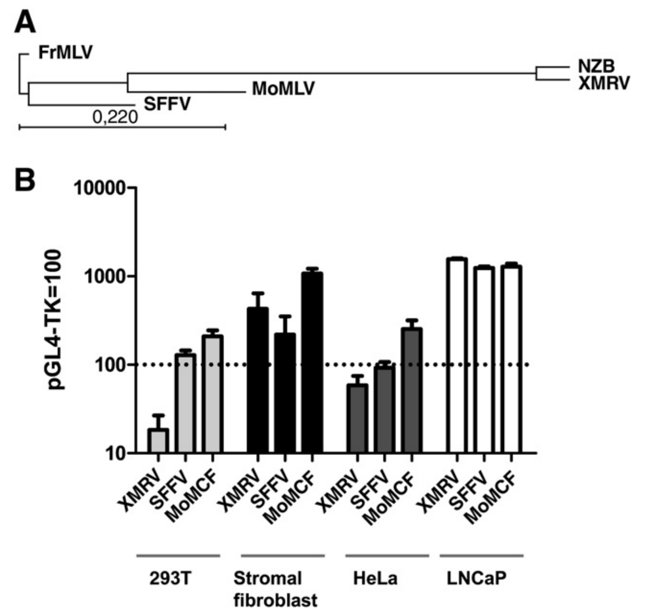

Fig. 5. (A) Phylogenetic comparison of U3-R LTR region of retroviruses. Phylogenetic tree based on U3-R nucleotide sequences from NZB gi|332081, XMRV gi|88765817, FrMLV gi|9626096, SFFV gi|9626955 and MoMLV gi|2801468. Multiple sequence alignment of the U3-R sequence were constructed with CLUSTALX and used to generate a neighbor-joining tree using the software CLC sequence viewer. Bootstrap values (n = 1000 trials) are shown as percentages. (B) XMRV–LTR activity shows minor differences to SFFV-LTR and MoMCF-LTR. The relative luciferase activity of U3-R-U5 reporter constructs of XMRV, SFFV and MoMCF was compared to each other in the cell lines indicated.

might influence receptor binding and virus entry, which has also been suggested for other retroviruses (Ghez et al., 2006; Jones et al., 2005; Pinon et al., 2003).

While receptor distribution does not explain XMRV restriction in vivo, promoter activity displays a preference for cells of the prostate compartment including stromal fibroblasts and established cancer cell lines LNCaP and DU145. In comparison to other investigated human cell lines, XMRV promoter activity shows up to 10-fold higher activity in stromal fibroblast and up to 30-fold increased activity in the prostate cancer cell line LNCaP, while the prostate cancer cell line DU145 displayed just a marginal increase when compared to HeLa and 293T cells. LNCaP, DU145 and prostatic stromal fibroblast do grow with similar proliferation rates while HeLa and 293T cells both process significantly higher proliferation rates. Increased LTR activity in prostate epithelium and prostate stromal fibroblasts is not unique to XMRV, as other related gammaretroviral promoters (i.e., Mo-MLV and SFFV) showed similar transcriptional activity: All cell lines tested demonstrated similar luciferase activity, with the exception of 293T cells, in which XMRV promoter was significantly less active compared to the other MLV-promoters.

Interestingly, we found efficient XMRV replication (as measured by viral RNA in dividing cells) in HeLa cells, in the prostate cancer cell lines DU145 and LNCaP, as well as in stromal fibroblasts isolated from prostate tissue. These results are partially in line with our observation of increased promoter activity in prostate cell lines. Interestingly, although 293T cells possess the lowest XMRV promoter activity, they are still capable of producing infectious viral particles–suggesting that other aspects of the virus life cycle are relative efficient in these cells.

Cells of the human rhabdomyosarcoma cell line TE671, and the human lung cancer cell line A549 can be efficiently infected with XMRV and display strong promoter activity (albeit significantly less than LNCaP cells), but they do not produce infectious XMRV particles; no viral particles were observed in concentrated cell culture supernatant nor could viral DNA be detected by PCR. These results demonstrate that after the initial step of infection, other blocks to XMRV infection affecting virus replication, virus integration, silencing, assembly or release can control virus spread.

In summary, we demonstrate here that in vitro XMRV efficiently infects not only prostatic stromal fibroblasts or prostate epithelial cells but also human cells of many different tissue types, as well as cells from various animal species. A putative in vivo restriction of XMRV infection cannot be explained by limited receptor expression or plasma membrane localization, as demonstrated by Western Blot analysis and binding experiments. These studies provide evidence for significantly higher promoter activity of gammaretroviral promoters in cells derived from prostate tissue and are in line with the in vivo observation of restricted XMRV infection to prostatic stromal fibroblasts as well as epithelial cells of the prostate. It will be of great interest to understand the mechanisms (and identify the cellular factors) by which XMRV infection/spread is controlled in vivo.

Material and methods

Cell culture

The cells used include the human cell lines TE 671 (ATCC# CCL-136), HeLa, 293T, the B-cell line Jijoye (ATCC #CCL-87), HL60 cells (ATCC #CCL-240), Reh cells (ATCC #CRL-8286), prostate cancer cell lines LNCaP (ATCC #CRL-1740) and DU145 (ATCC #HTB-81). Cell lines from non-human species include the feral mouse cells SC-1 (ATCC#CRL-1404), murine NIH3T3 fibroblasts, hamster ovary CHO (ATCC #CCL-61), hamster A23 and BHK cells, porcine PK15 cells, dog D17 cells, mink CCL64 cells, monkey COS7, as well as bovine MDBK cells. Suspension cell lines, HeLa cells, HL60 cells and LNCaP cells were grown in RPMI medium (Invitrogen) supplemented with 10% FCS. All other cell lines were kept in DMEM (Invitrogen) supplemented with 10% FCS and grown at 37 °C, 5% CO_2 and 100% relative humidity.

Stromal cell lines (PrSc) were established as described (Gerdes et al., 1996; Tuxhorn et al., 2002). Briefly, fresh tissue cores were minced into 1-mm cubes, washed with HBSS buffer and put into 24-well plates containing DMEM supplemented with 10% FCS, 5 μg/ml of insulin and 0.5 μg/ml of testosterone. The explants were incubated at 37 °C, 5% CO_2 and 100% relative humidity. Medium was changed every 48 h. Stromal cells migrated out of the tissue and attached to the culture dish. Standard immunocytochemistry procedures were used to evaluate the cell phenotype. Cytokeratin expression was negative with the Santa Cruz pan-cytokeratin Ab sc-8018 and all cell lines were 100% vimentin positive (sc-7557, Santa Cruz Biotechnology). HUVEC cells were maintained in endothelial cell basal medium (Cambrex, Bioscience) supplemented with 10% FCS and EGM Single Quots (Cambrex Bioscience). Only cells maintained for 5 to 10 passages were used in experiments.

Cells transiently expressing the XPR1 receptor were generated by calcium phosphate-mediated transfection of receptor expression constructs, pcDNA3.1-hXPR1 (Yang et al., 1999). 24 h post transfection cells were trypsinized and seeded at 1:10 dilution into medium containing G418 and selected for 7–10 days. Stable hXPR1 expressing cell lines were generated by single-cell clone selection. Briefly, serial tenfold dilution of transfected cells was seeded into 96-well plates 10 days after transfection. Colonies in individual wells of plates in which approximately 20% of the total wells showed outgrowth after 2 weeks were considered to have arisen from a single cell clone.

Plasmids

Env sequences from XMRV VP62 (GenBank: DQ399707) were obtained by PCR using primers with BamHI restriction sites (Env 5′ BamHI 5-*GGATCC*ATGGAAAGTCCAGCGTTCTC-3 and Env 3′ BamHI 5-*GGATCC*GTAGCTAGCGTGCTAAGCC-3′) and XMRV clone A0H4 (Urisman et al., 2006) as template. PCR fragments were temporarily cloned into pCR2.1 by TA cloning technique (Invitrogen), excised using BamHI and ligated into the expression construct pHCMV (Yee et al., 1994). The resulting plasmid, pHCMV-env VP62 was confirmed by sequencing. Plasmid pCSI-ENZB expressing the env protein of the xenotropic NZB MLV has been described before (Battini et al., 1999). Expression constructs encoding for 10A1 env protein (pHCMV-10A1env) was constructed by PCR amplification using pRR151 (Ott et al., 1990) as template and insertion of the PCR fragment in BamHI sites of pHCMV. Ecotropic MLV (env protein pEnv-IRES-puro) was used as an additional control (Miller and Chen, 1996; Morita et al., 2000). Plasmids used in pseudotyping experiments pSF91-I-eGFP-PRE and pSV-MoMLVGag-pol have been published earlier (Beyer et al., 2002; Schwieger et al., 2002).

LTR sequences from XMRV VP62, spleen focus forming virus (SFFV) and Moloney MLV were amplified by PCR using a proof reading taq enzyme (Platinum PfxDNA Polymerase Invitrogen), the upstream primer U3_LTR_XhoI (5′-GCTCGAGGTAACGCCATTTTGC-3′) and the downstream primer U5_LTR_HindIII (5′-GCAAGCTTAATGAAA-GACCCCC-3′) Plasmids serving as PCR templates were pSF1 (GenBank accession number AJ224005) containing the 0.58-kbp LTR of spleen-focus forming virus and pBMN-Z-IN (Addgene plasmid 1735) containing the 0.62 kbp moloney MLV-LTR sequences. All PCR fragments were cloned into the *firefly* luciferase reporter plasmid pGL4.10 [luc2] (Promega) and subsequently sequenced.

XMRV proviral clone was constructed fusing two overlapping fragments VP62 AM-2-9 and VP62 AO-H-4, which have been described earlier (Dong et al., 2007; Urisman et al., 2006), pCR.2.1 TOPO (Invitrogen) was used as vector backbone.

XMRV env fusion protein pCSI-XMRV-RBD-rFc

A pCSI expression vector (Battini et al., 1999) containing the receptor binding domain of XMRV env (aa 1-283) up to the proline rich region (PRR) and fused to a rFc at its carboxy terminus has been kindly provided to us by JL Battini and M Sitbon (IGMM-CNRS, Montpellier). Recombinant protein was produced by transfecting 293 cells and harvesting the $XMRV_{RBD}$rFc containing supernatant 48 h after transfection. The supernatant was filtered (0.45 µm) and stored at −80 °C. Protein expression was confirmed by Western blotting.

Immunoblotting and antibodies

25 µg of total protein was analyzed by SDS PAGE and immunoblotted using polyclonal rabbit antisera against XPR1 (Abcam). Equal amounts of protein loaded in cell lysate immunoblotting experiments were verified by incubation with anti-β-tubulin Ab (Santa Cruz) or actin Ab 1501 (Chemicon). Concentrated supernatant from the hybridoma cells CRL-1912 (ATCC) was used to detect p30-Gag by immunoblotting experiments.

Transient production of retrovirus vector pseudotypes and infection protocol

Replication incompetent Env pseudotyped retroviral particles were produced by transient transfection of phoenix cells (provided by the Nolan lab, Stanford University, CA). 5×10^6 cells were seeded in 10-cm dish 12 h pre transfection. Culture medium was replaced shortly before transfection with DMEM/FCS. 5 µg of pSF91-I-eGFP-PRE (Schwieger et al., 2002), 10 µg of pSV-Mo-MLVgagpol (Beyer et al., 2002) and 5 µg of the Env expressing construct were transfected using $CaPO_4$-HBS technique according to manufacturer's instructions (Profection mammalian transfection system, Promega). Medium was changed 6 h after transfection with 6 ml of DMEM/FCS containing 20 mM HEPES. Supernatant was collected every 12 h. Supernatant was passaged through a 0.2-µm pore size filter, aliquoted and frozen at −80 °C.

All viral supernatants were titered on TE 671 or SC-1 cells to determine the MOI (multiplicity of infection) as described previously (Beyer et al., 2002). Briefly, 5×10^4 cells seeded in 24-well plate 4 h pre infection were incubated with the viral supernatant in the presence of 8 µg/ml of polybrene and centrifuged at 800 × g at 37 °C for 1 h. Medium change was performed 1 day later and retroviral titers were determined by flow cytometry 3 days post infection and expressed as GFP iU/ml.

Infection using replication competent XMRV

XMRV proviral DNA was transfected into LNCaP cells or 293T cells to produce virus containing supernatant as described earlier (Dong et al., 2007). Cells seeded in 12-well or 24-well plates were infected with XMRV containing supernatant in the presence of polybrene. Polybrene containing virus-supernatant was removed 4 h after infection and replaced by fresh medium. Cells were subsequently cultured and successful infection was monitored by RT-PCR as well as Western blotting for viral proteins.

Binding assay

The pCSI-XMRV-RBD-rFc construct was transiently expressed in 293T cells; 72 h after transfection culture supernatant containing the recombinant $XMRV_{RBD}$rFc protein was collected, filtrated and used in binding experiments. Target cells were washed in PBS without $Ca^{2+}$and Mg^{2+}, detached using PBS/EDTA and washed again with PBS containing 2% FCS. 5×10^5 cells were incubated 30 min at 37 °C with 500 µl of culture medium containing $XMRV_{RBD}$rFc. Cells were washed multiple times with PBS containing 2% FCS and incubated with an anti-rabbit-FITC antibody (Santa Cruz) for 30 min at 37 °C. Cells were analyzed by flow cytometry.

Transient transfections and luciferase assay

250 ng of plasmid DNA (200 ng of reporter plasmid containing the firefly luciferase gene and 50 ng of renilla luciferase plasmid (pBIND-Renilla, Promega) was transiently transfected in 5×10^4 293 cells in 6-well plates according to manufactory instructions (ProFection mammalian transfection system, Promega). Cells were lysed 24 h after transfection and luciferase activity was determined using the dual luciferase reporter assay system (Promega E1960) and the Infinite M200 microplate reader (Tecan). Activity of the LTR reporter construct was calculated as the percentage activity relative to a plasmid containing the widely active herpes simplex virus TK promoter in front of the *firefly* luciferase gene, pGL4 [luc2/TK] (Promega).

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