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Evaluation of Cellular Determinants Required for In Vitro Xenotropic Murine Leukemia Virus-Related Virus Entry into Human Prostate Cancer and Noncancerous Cells

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The newly identified retrovirus—the xenotropic murine leukemia virus-related virus (XMRV)—has recently been shown to be strongly associated with familial prostate cancer in humans (A. Urisman et al., PLoS Pathog. 2:e25, 2006). While that study showed evidence of XMRV infection exclusively in the prostatic stromal fibroblasts, a recent study found XMRV protein antigens mainly in malignant prostate epithelial cells (R. Schlaberg et al., Proc. Natl. Acad. Sci. U. S. A. 106:16351–16356, 2009). To help elucidate the mechanisms behind XMRV infection, we show that prostatic fibroblast cells express Xpr1, a known receptor of XMRV, but its expression is absent in other cell lines of the prostate (i.e., epithelial and stromal smooth muscle cells). We also show that certain amino acid residues located within the predicted extracellular loop (ECL3 and ECL4) sequences of Xpr1 are required for efficient XMRV entry. Although we found strong evidence to support XMRV infection of prostatic fibroblast cell lines via Xpr1, we learned that XMRV was indeed capable of infecting cells that did not necessarily express Xpr1, such as those of the prostatic epithelial and smooth muscle origins. Further studies suggest that the expression of Xpr1 and certain genotypes of the RNASEL gene, which could restrict XMRV infection, may play important roles in defining XMRV tropisms in certain cell types. Collectively, our data reveal important cellular determinants required for XMRV entry into different human prostate cells in vitro, which may provide important insights into the possible role of XMRV as an etiologic agent in human prostate cancer.

Prostate cancer is the most common male malignancy in Western countries and the second most common cause of cancer-related deaths in males worldwide (15, 24). The known risk factors for prostate cancer are hormones (i.e., androgens), diet, sex, and race, as well as environmental and genetic factors (27). A recent study suggests that susceptibility to prostate cancer can be influenced by the genetic variations associated with an antagonistic coevolution, which occurs between a specific host locus (RNASEL), known to be involved in antiviral innate immune defense, and a viral pathogen (38). Indeed, several epidemiologic studies have supported the involvement of the RNASEL gene in the prostate cancer etiology (4, 5, 30, 31), whereas other studies do not (9, 22, 34, 43). Some studies have reported that individuals with a single mutated copy of the RNASEL gene have a 50% increased risk for prostate cancer, whereas those with homozygous mutant RNASEL alleles have a 2-fold-increased risk of prostate cancer (5).

The RNASEL gene encodes for the RNase L protein, a constitutively expressed latent endoribonuclease, which mediates the interferon-inducible 2-5A system against viral and/or cellular double-stranded RNAs (8, 16, 20, 23, 49, 50). The RNase L “Q” variant allele (R462Q) shows a 3-fold decrease in catalytic activity compared to the wild-type enzyme (5, 44). The possible association of mutant RNASEL alleles with human prostate cancers suggests an enhanced susceptibility of prostate tissues to a viral agent. This hypothesis has led to the recent identification of a new human retrovirus, xenotropic murine leukemia virus (MuLV)-related virus (XMRV), in 40% of prostate cancer patients with the QQ variant alleles of RNASEL compared to 1.5% among heterozygous (RQ) and wild-type (RR) RNASEL carriers (41). XMRV virus infection appears to be susceptible to inhibition by interferon and its downstream effector RNase L protein (7). However, a recent study has provided some evidence to show that XMRV infection is independent of the RNASEL genotype (34), suggesting that population differences and/or other environmental or genetic factors may influence the impact of RNASEL on prostate cancer development.

The XMRV genome is 8,185 nucleotides in length and shares up to 95% overall nucleotide sequence identity with known xenotropic MuLVs (41). One receptor for xenotropic MuLVs is Xpr1, a 696-amino-acid protein with multiple transmembrane-spanning domains (2). Expression of this protein in Chinese hamster ovary (CHO) cells that are not known to express Xpr1 endogenously confers an enhanced susceptibility of these cells to xenotropic MuLV infection (2). Infection of hamster and mouse cells with XMRV-like virus that is derived from a prostate cancer cell line (22Rv1) also requires Xpr1 as a receptor (18). Earlier studies have demonstrated the importance of certain residues located within the putative third and fourth extracellular loops (ECL3 and ECL4) of Mus dunni’s Xpr1 in conferring infection by xenotropic MuLVs (25). Furthermore, it has been shown that the specific and common

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receptor determinants for xenotropic and polytropic murine retroviruses are simultaneously present in discrete domains of a single Xpr1 protein (42). In the present study, we characterized for the first time the important molecular determinants on Xpr1 required for XMRV infection and investigated the role of RNase L in restricting XMRV infection of various human prostate cancer and noncancerous cell lines.

MATERIALS AND METHODS

Plasmids, cell lines, and viruses. The pLXSN-Xpr1 plasmid carrying the XPR1 gene was kindly provided by D. Miller (University of Washington). The prostate cancer cell lines DU145, PC-3, and LNCaP were used for XMRV infection. All cells were originally obtained from the American Type Culture Collection and cultured in conditions as recommended by the supplier. The hTERT immortalized fibroblast (P1197T), epithelial (Ep156T), and smooth muscle (Pm151T) cell lines were provided by V. Rotter (Weizmann Institute of Science, Israel) and cultured in conditions as recommended (19). The human primary (nonimmortalized) prostate fibroblast cells isolated from normal (Pt-N) or cancerous (Pt-C) tissues of prostate cancer patients were kind gifts of L. Chung (Emory University) and were cultured as described previously (39). The primary prostate epithelial cell line (PrEC) was purchased from Lonza (Walkersville, MD) and cultured in conditions as recommended by the supplier. The Chinese hamster ovary cell line (CHO) lacking endogenous Xpr1 expression and showing resistance to XMRV virus infection was used as a negative control in all experiments. All cell lines were maintained in RPMI medium (Invitrogen).

DU145-C7 cells, which carry integrated copies of XMRV and constitutively produce wild-type XMRV virus, was kindly provided by R. Silverman (Cleveland Clinic) and grown in RPMI medium (7, 41). Supernatant collected from the cells showing resistance to XMRV virus infection was used as a negative control in all experiments. The immortalized epithelial (Ep156T) and primary cell lines were infected with XMRV-pseudovirus or wild-type XMRV virus in the presence of 25 mM chloroquine, with 14 μg/ml for Pm151T in the presence of 8 μg/ml of Polybrene and 10 μg/ml for the immortalized cell lines, and 10 μg/ml of Polybrene/ml and in their respective medium was replaced with a fresh aliquot of the medium, and the culture was extended at 72°C for 2 min. The 2.1-kb protein production was purified with a QIAquick gel extraction kit (Qiagen), cloned into the expression vector pcDNA3.1-Intron A, and sequenced. The QuikChange PCR mutagenesis protocol (Stratagene) was used to generate the Xpr1 mutants E338Q (K500E, Δ582T). The forward and reverse primers used to make the K500E mutant construct were 5’-GCCCCTTTACAGCACTCACGAGGAACGAGGTCACTCG-3’ and 5’-CCGAGTGACCTCGTTCCTCGTGAGTGCTGTAAAGGGC-3’, respectively. The primers used for the deletion at position 582 were 5’-CAATACCTGATTCATCCTCTCTTTGTCCTC-3’ and 5’-GAGGCGAACTTGAAGGATAATCGGATTGTTG-3’. The wild-type and mutant Xpr1 constructs were transfected in CHO cell line, which was found to be infection with the XMRV-pseudovirus as described above. XPR1 gene expression was analyzed by Western blotting after 48 h posttransfection with either 1 μg/ml a blotting was performed with anti-EHA antibody (Santa Cruz) as described in more detail below or by reverse transcription-PCR (RT-PCR). To perform RT-PCR, total RNAs were isolated from the transfected cell lines and used to transcribe Xpr1 cDNA by using Superscript II (Invitrogen), which was followed by PCR amplification with GoTaQ DNA polymerase (Promega) for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C, and followed by final 10 min at 72°C in a reaction that contained 200 μM of dNTPs, 2 μg of DNA, 20 ng of primer, 7 μg of the pLXSN-Xpr1 plasmid expressing hemagglutinin (HA)-tagged Xpr1 gene and its mutagenesis.

Construction of the plasmid expressing hemagglutinin (HA)-tagged Xpr1 gene and its mutagenesis. To create the HA tag version of Xpr1, PCR was performed to amplify the Xpr1 coding region using 2 μl of the pLXSN-Xpr1 plasmid as the template in a 50-μl PCR that contains 1 U of high-fidelity Vent DNA polymerase (NEB) and 0.2 μM concentrations of the primers Expr1-HA forward (5’-ATGCAAGGTCCTGGAGGATGAACTTGGCCG-3’) and Expr1-HA reverse (5’-ATGCG GCAGCGCTCAAGGTAATCCTGGAACATCTGTTAATAGTATGCGTGTCATCATC-3’). After denaturation at 94°C for 4 min, the reaction proceeded with 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. The 2.1-kb protein production was purified with a QIAquick gel extraction kit (Qiagen), cloned into the expression vector pcDNA3.1-Intron A, and sequenced. The QuikChange PCR mutagenesis protocol (Stratagene) was used to generate the Xpr1 mutants E338Q (K500E, Δ582T). The forward and reverse primers used to make the K500E mutant construct were 5’-GCCCTTTACAGCACTCACGAGGAACGAGGTCACTCG-3’ and 5’-CCGAGTGACCTCGTTCCTCGTGAGTGCTGTAAAGGGC-3’, respectively. The primers used for the deletion at position 582 were 5’-CAATACCTGATTCATCCTCTCTTTGTCCTC-3’ and 5’-GAGGCGAACTTGAAGGATAATCGGATTGTTG-3’. The wild-type and mutant Xpr1 constructs were transfected in CHO cell line, which was found to be infection with the XMRV-pseudovirus as described above. XPR1 gene expression was analyzed by Western blotting after 48 h posttransfection with either 1 μg/ml a blotting was performed with anti-EHA antibody (Santa Cruz) as described in more detail below or by reverse transcription-PCR (RT-PCR). To perform RT-PCR, total RNAs were isolated from the transfected cell lines and used to transcribe Xpr1 cDNA by using Superscript II (Invitrogen), which was followed by PCR amplification with GoTaQ DNA polymerase (Promega) for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C, and followed by final 10 min at 72°C in a reaction that contained 200 μM of dNTPs, 2 μg of DNA, 20 ng of primer, 7 μg of the pLXSN-Xpr1 plasmid expressing hemagglutinin (HA)-tagged Xpr1-R (5’-AGTTGTACCTGTATCCATGTTGTTACATCTC-3’) primers. In addition, a set of universal primers was used to PCR amplify the β-actin gene as a control.

siRNA knockdown of XPR1 and desmoglein-2 genes. DU145 cells at approximately 50 to 60% confluence were grown in 12-well plates for 24 h before transfection with small interfering RNA (siRNA). Pd esiRNAs (ON-TARGETplus SMART pool siRNA reagent for XPR1 and control siRNA) were purchased from Dharmacon, Inc. The SMART pool siRNAs against XPR1 (catalog no. L-005752-00) were 5’-ACACUAAAGGUAAUUGAUAGA-3’, 5’-UCGAUAUCUCUAACAUCUUC-3’, 5’-GGCUUGCCGUGUAUUAUUAA-3’, and 5’-GGCGCUUUCUCUAUAGAUUU-3’, and the control siRNA was ON-TARGET plus SMART pool siRNA reagent for XPR1 and control siRNA were purchased from DharmaFECT transfection reagent 1. Cells were incubated with the transfection medium for 48 h and infected with XMRV pseudovirus. Cells infected with pseudovirus were analyzed by fluorescence microscopy and flow cytometry using the FACS caliber (BD Biosciences) as described above. The levels of XPR1 gene knockdown were detected by RT-PCR using the primer sets as described above.

RT-PCR and quantitative real-time RT-PCR. Since none of the commercial polyclonal antibodies (2G8 from Abnova, N-19 from Santa Cruz, and ab13291 from Abcam) proved to be effective at detecting Xpr1 expression by Western blotting or IHC (data not shown), we decided to use RT-PCR and quantitative real-time RT-PCR for this purpose. To this end, total cellular RNAs were isolated from different cell lines, treated with RNasefree DNase (Promega), and extracted with acid phenol-chloroform (5:1) to remove all RNase. PURIFIED RNAs were used as templates to synthesize Xpr1 cDNAs by using Superscript II (Invitrogen), which were then amplified by PCR using the GoTaq DNA polymerase (Promega) at 94°C for 4 min; followed by 30 cycles at 94°C, 30 s at 58°C, and 45 s at 72°C for 30 cycles; and finally by 10 min at 72°C in a reaction that contained the XPR1out-F (5’-CAGTGGTTTACCGGCTG-3’) and XPR1out-R (5’-GCAAATGCCTGAGATGAGG-3’) primers as described previously (7). In addition, a set of universal primers (Table S2) were used to PCR amplify the β-actin gene as a control. Quantitative real-time RT-PCR was performed using Sybr green dye mix (Invitrogen) and β-actin as an internal control in a 20-μl reaction mixture containing the primers Xpr1-F (5’-CGTAAAGAGAGCGGAGG-3’) and Xpr1-R (5’-GCAAACAGGTGATGAGG-3’) spe-
cific for Xpr1. The PCR condition was 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The RNA levels, expressed as threshold cycle (Ct) values, were normalized by the β-actin RNA level. RT-PCRs to amplify the XMRV spliced env RNA in cell lysates of infected prostate cancer, immortalized, and primary cell lines were conducted by treatment with GoTaq polymerase (Promega) for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1.5 min at 72°C; and finally by 10 min at 72°C using the forward primer at the expected spliced site at nucleotide (nt) 195 (5'-ACCGTCGGGAG/GCCCTCCAAGCAG-3') and reverse primer at nt 8185 (5'-TTGCAACACAGGAAAAGCTTTATTGG-3').

**Western blotting.** Rat monoclonal antibody to spleen focus-forming virus (SFFV) Gag was prepared from R187 cells (American Type Culture Collection) (6). Monoclonal antibodies to α-actin and the HA tag were purchased from Sigma and Santa Cruz Biotech, respectively. Monoclonal antibodies against vimentin, cytokeratin 8, and myosin II LC were kindly provided by A. Nusrat and L. Chung (Emory University). Proteins in cell extracts (20 to 100 μg) were separated in 10 or 12% polyacrylamide-sodium dodecyl sulfate gels, depending on the expected molecular masses of the proteins, and transferred onto polyvinylidene difluoride transfer membrane. Blots were blocked with TBS (Tris-base saline) containing 0.1% Tween (TBS-T) and 5% dried milk for 1 h and then incubated with primary antibodies against Gag, vimentin, cytokeratin 8, myosin II LC, HA tag, or α-actin in 2.5% TBS-T at 4°C overnight with shaking. The blots were washed three times with TBS-T and were further incubated with appropriate secondary antibodies: anti-mouse IgG-HRP for vimentin; cytokeratin, myosin II LC, HA tag, or α-actin in 2.5% TBS-T at 4°C overnight with shaking. After four washes with TBS-T, the protein bands were visualized by using enhanced chemiluminescence detection reagents (Amersham Pharmacia).

**Genotype.** All of the telomerase-immortalized primary prostate cell lines used in the present study were genotyped for the R462Q (1385G→A) RNAseL variant using a premade TaqMan genotyping assay (Applied Biosystems). Portions (5 ng) of genomic DNA from each of the cell lines were assayed according to the manufacturer's instructions and analyzed on an Applied Biosystems 7900HT fast-real-time PCR system instrument.

**RESULTS**

**Xpr1 as a receptor for XMRV infection.** Previous studies have identified two critical amino acid residues in the extracellular loops (ECLs) of the murine Xpr1 protein, K500 in ECL3 and T582 in ECL4 loop, either of which alone could mediate entry of the xenotropic murine leukemia viruses (XMLVs) (25, 42). Yan et al. defined the specific determinants on Xpr1 protein required for entry of the different types of murine retroviruses (i.e., XMLV, polytropic PMLV, and the wild mouse virus CasE#1) into mouse cells derived from evolutionary divergent species of the Asian mouse species *Mus pahari* and the laboratory strain NIH 3T3 (46). These authors showed that the K500 residue on the ECL3 is the entry determinant for PMLVs, the T582 residue on the ECL4 is required for CasE#1 entry, but that both residues in the ECL3 and ECL4 are required for XMLV entry into cells (46). Whereas both of these amino acid residues can independently mediate entry of XMLVs, they are, however, not functionally equivalent, since the T582 but not K500 can function as a receptor for the wild mouse CasE#1, while K500 but not T582 mediates efficient entry of the xenotropic AKR6 virus (25, 46). A related study showed that the combination of a substitution mutation in the ECL3 and a single threonine (Thr) deletion at position 582 of the ECL4 of the Xpr1 protein could completely inhibit XMLV infection (25).

Since the molecular determinants on Xpr1 protein required for XMRV infection had never been mapped, we introduced the same mutations at both the ECL3 and ECL4 loops into the Xpr1 expression vector and transfected this mutant construct (Xpr1-M) or the wild-type Xpr1 vector into CHO cell line, which lacks endogenous Xpr1 expression. XMRV-pseudovirus expressing the *GFP* gene was used to infect these cells. Cells expressing the wild-type *XPR1* gene, as detected by RT-PCR and Western blotting (Fig. 1B and 1C), were expectedly susceptible to XMRV-pseudovirus infection, whereas those expressing the mutant Xpr1 showed no evidence of viral infection (Fig. 1A), demonstrating for the first time the requirement of the ECL3 and ECL4 loops of Xpr1 for XMRV infection. As controls, DU145 prostate cancer cells, which are known to express the Xpr1 protein, were susceptible to XMRV infection, whereas CHO cells with no endogenous expression were not susceptible (Fig. 1A).

**Differential expression of Xpr1 in prostate cancer and immortalized noncancerous cell lines.** XMRV viral genomic materials and protein antigens have been detected, respectively, by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) in the stromal fibroblast cells of human prostate tissues collected from patients with prostate cancer (7, 41). We reasoned that the susceptibility of cells to XMRV infection must be dependent upon the proper expression of the Xpr1 receptor on the target cells. We therefore attempted to detect *XPR1* expression in several prostate cancer and noncancerous cell lines by RT-PCR and/or quantitative real-time RT-PCR. These methods were chosen because none of the commercially available polyclonal antibodies (2G8 from Abnova, N-19 from Santa Cruz, and ab13291 from Abcam) proved to be effective at detecting Xpr1 expression by Western blotting.
and/or IHC (data not show). We used a series of telomerase-immortalized primary cell lines of the epithelial (Ep156T), smooth muscle (Pm151T), and fibroblast (Pf179T) origins that were previously derived from healthy human prostate glands (19). In addition, we also used nonimmortalized fibroblast cell lines, Pt-N and Pt-C, which were derived from benign and malignant prostate tissues, respectively (39), and the established primary prostatic epithelial PrEC cells (Lonza). Even though most of the telomerase-immortalized cell lines used in the present study, except for the Pf179T, have been characterized (19), we validated the cell-type origins of these and most other primary cell lines used in the present study by determining the expression of the specific protein markers by Western blot analysis with the appropriate antibodies. As expected, the epithelial cells (Ep156T and DU145) expressed cytokeratin 8 (Fig. 2A), whereas all fibroblast cells (Pf179T, Pt-N, and Pt-C) highly expressed vimentin (Fig. 2B and data not shown), and the smooth muscle cells (Pm151T) expressed myosin II LC protein (Fig. 2C), correlating with their respective cell-type origins. To detect the expression of Xpr1 transcript, we used several prostate cancer cell lines (DU145, LNCaP, and PC3) as positive controls and CHO cells as a negative control. Xpr1 transcripts were expressed in all prostate cancer cell lines and in all primary fibroblast cell lines tested, but were uniquely absent in the immortalized prostate epithelial (Ep156T) and smooth muscle (Pm151T) cells (Fig. 3A and B). We also found minimal to no expression of XPR1 in the primary prostatic cell line PrEC as judged by RT-PCR (Fig. 3C). However, we have not determined whether different versions (e.g., isoforms) of Xpr1 are expressed in these cells that can be used by XMRV as its alternative receptors. Overall, these data indicate that XPR1 gene expression is cell type dependent, which may explain why XMRV viral nucleic acid and protein can only be detected in the fibroblast cells of the prostate as reported previously (41).

Susceptibility of prostate cancer and noncancerous cell lines to XMRV-pseudovirus infection. We next sought to determine whether Xpr1 expression correlates with XMRV susceptibility. XMRV-pseudoviruses expressing the GFP gene were used to infect the above-described cell lines that have been examined for Xpr1 expression. At 72 h postinfection, we found that all prostate cancer cell lines tested, which expressed Xpr1, were susceptible to XMRV-pseudovirus infection, whereas CHO cells with no endogenous Xpr1 expression were not (Fig. 4A and F). Likewise, we showed that all fibroblast cell lines tested, which expressed high levels of XPR1 gene, were also susceptible to XMRV-pseudovirus infection (Fig. 4B and F), a finding consistent with earlier findings on detection of XMRV viral antigens in primary prostatic tissues (41). Surprisingly, we detected low levels of XMRV-pseudovirus infection in immortalized epithelial (Ep156T) and smooth muscle (Pm151T) cell lines, as well as the primary prostatic epithelial (PrEC) cell line (Fig. 4C, D, E, and F), despite the fact that they did not express endogenous Xpr1 as judged by RT-PCR and/or quantitative real-time RT-PCR (Fig. 3B and C and Fig. 4G). Upon Xpr1 overexpression by transient transfection as evidenced by Western blot analysis (Fig. 4H), the epithelial cells (Ep156T) showed marked increase in XMRV-pseudovirus infectivity ($P < 0.0001$), but the smooth muscle cells (Pm151T) showed no appreciable difference (Fig. 4C, D, E, and F), the implications for which are discussed below.

In an effort to confirm prior studies on the requirement of XPR1 expression for XMRV infection (7, 12), we used siRNA technology to reduce the endogenous XPR1 gene expression in DU145 prostate cancer cells (Fig. 5A), which effectively diminished entry of the XMRV-pseudovirus ($P < 0.0002$) into these treated cells, whereas those treated with a scrambled siRNA as
a negative control did not (Fig. 5C and D), providing strong evidence for Xpr1 as the main receptor of XMRV in these cells. On the contrary, there is no significant difference in the levels of XMRV-pseudovirus infection of the Xpr1 siRNA-treated primary epithelial cells Ep156T compared to those treated with a scrambled siRNA (Fig. 5C and D), suggesting alternative mode(s) of XMRV entry in these cells. As a control for the siRNA knockdown, we similarly transfected the Ep156T cells with siRNA that targets the conserved region of the epithelial cell specific desmoglein-2 gene, which is a member of the cadherin superfamily of genes that mediate adhesion of intercellular junctions (i.e., desmosomes) (36), and showed significant knockdown of this gene (Fig. 5B), implying that the Ep156T primary epithelial cells were indeed susceptible to siRNA transfection and knockdown.

**Susceptibility of prostate cancer and noncancerous cell lines to wild-type XMRV infection.** We next sought to determine whether the cell lines tested above were also susceptible to wild-type XMRV viruses, which were generated from DU145-C7 cells that carry integrated copies of the XMRV viral genome, and hence,
In this report, we show that the receptor (Xpr1) sufficient for XMRV infection is expressed in human prostate stromal fibroblast cell lines but absent in other cell lines derived from the prostate gland, which may help explain previous observations that XMRV viral antigens are present in the stromal fibroblasts of prostate tissues and of peripheral blood collected from cancer patients (41). In addition, we found evidence for XMRV infection of cells that appear not to express detectable levels of XPR1 by RT-PCR assaying, indicating other possible entry pathway(s) for XMRV (34). We also define the molecular determinants of Xpr1 that are required for efficient XMRV infection.

The Xpr1 receptor has been characterized as a transmembrane protein of unknown function, although it shows some homology to yeast genes involved in signal transduction and phosphate transport (2, 40, 48). Previous studies have implicated Xpr1 extracellular loop sequences (ECL3 and ECL4) in mediating entry of the XMLVs. Specifically, two amino acids, K500 in the ECL3 and T582 in ECL4, have been found to be most essential for Xpr1 function as a viral receptor by directly interacting with the viral envelope protein (25, 46). Our study demonstrates for the first time the significance of these two specific amino acids in determining susceptibility to XMRV infection (Fig. 1), validating previous findings that this novel human retrovirus also utilizes Xpr1 as a major receptor for viral entry (46). Recently, Yan et al. screened a panel of polytropic and xenotropic murine retroviruses (X/PMVs), including a novel cytopathic XMV-related virus (Cz524) and XMRV, for infectivity of rodent cells that carried various polymorphic Xpr1 receptors (47). Using a panel of Xpr1 mutants and chimeras, they identified five critical amino acids in ECL3 and ECL4, including E500 and T582, as well as K500, T507, and V508, required to mediate efficient entry of these different viruses (47). It remains to be determined whether these other residues in ECL3 and ECL4 besides K500 and T582 are absolutely required for XMRV entry.

A common mechanism for virus-induced cellular transformation is activation of proto-oncogenes or inactivation of tumor suppressor genes as a consequence of proviral insertions into the host genome (10). Recent studies have provided evidence for integration of XMRV into the genomes of prostate tissues of cancer patients (7, 17), as well as into the 22Rv1 prostate carcinoma cell line (18). However, evidence for common XMRV integration sites within or near proto-oncogenes or tumor suppressor genes in tumor tissues is still lacking (17). FISH and IHC assays have revealed evidence for XMRV infection in the prostate stromal fibroblastic and hematopoietic cells, rather than in other cell types of the prostate (41), arguing that any effect of XMRV on prostate cancer development might involve alterations of the tumor microenvironment.

In search of an explanation for the differential XMRV infectivity of the various prostate cell types, we screened XPR1 gene expression in several prostate cancer and noncancerous cell lines derived from various prostate cell types. By RT-PCR, we found that all of the prostate cancer cell lines, telomerase-immortalized and primary fibroblast cell lines that were isolated from either benign (Pt-N) and malignant (Pt-C) areas of prostate gland, which may help explain previous observations that XMRV viral antigens are present in the stromal fibroblasts of prostate tissues and of peripheral blood collected from cancer patients (41). In addition, we found evidence for XMRV infection of cells that appear not to express detectable levels of XPR1 by RT-PCR assaying, indicating other possible entry pathway(s) for XMRV (34). We also define the molecular determinants of Xpr1 that are required for efficient XMRV infection.

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A common mechanism for virus-induced cellular transformation is activation of proto-oncogenes or inactivation of tumor suppressor genes as a consequence of proviral insertions into the host genome (10). Recent studies have provided evidence for integration of XMRV into the genomes of prostate tissues of cancer patients (7, 17), as well as into the 22Rv1 prostate carcinoma cell line (18). However, evidence for common XMRV integration sites within or near proto-oncogenes or tumor suppressor genes in tumor tissues is still lacking (17). FISH and IHC assays have revealed evidence for XMRV infection in the prostate stromal fibroblastic and hematopoietic cells, rather than in other cell types of the prostate (41), arguing that any effect of XMRV on prostate cancer development might involve alterations of the tumor microenvironment.

In search of an explanation for the differential XMRV infectivity of the various prostate cell types, we screened XPR1 gene expression in several prostate cancer and noncancerous cell lines derived from various prostate cell types. By RT-PCR, we found that all of the prostate cancer cell lines, telomerase-immortalized and primary fibroblast cell lines that were isolated from either benign (Pt-N) and malignant (Pt-C) areas of prostate gland, which may help explain previous observations that XMRV viral antigens are present in the stromal fibroblasts of prostate tissues and of peripheral blood collected from cancer patients (41). In addition, we found evidence for XMRV infection of cells that appear not to express detectable levels of XPR1 by RT-PCR assaying, indicating other possible entry pathway(s) for XMRV (34). We also define the molecular determinants of Xpr1 that are required for efficient XMRV infection.
the prostate gland of cancer patients (39) expressed high levels of Xpr1 transcripts, which were completely absent in the benign prostatic epithelial and smooth muscle cell lines (Fig. 3B and 4G). These results are consistent with an earlier observation, which shows XMRV infection in the prostate fibroblasts but not in the epithelial cells of the prostate tissues (41). All cell lines that expressed the $XPR1$ gene indeed were susceptible to XMRV infection (Fig. 4A, B, and F). In addition, we observed some levels of XMRV infection in the prostate epithelial and smooth muscle cell line even though they appeared to lack Xpr1 expression (Fig. 4C to G). Likewise, we found minimal to no expression of Xpr1 in PrEC cells by the RT-PCR method, which corresponded to relatively low XMRV-pseudovirus infectivity of these cells (32).

In a recent study, XMRV protein antigens have been detected mainly in malignant prostatic epithelial cells (23%) but only rarely in stromal cells (4%) of prostate cancer tissues collected from one institution (34). The expression of Xpr1 in the XMRV-positive malignant epithelial cells noted in that study is unknown, however. It is important to note that all prostate cancer cell lines used in the current study (DU145, LNCaP, and PC3) are of the epithelial origin, which curiously express high levels of the $XPR1$ gene, and as such are readily susceptible to XMRV infection (Fig. 3A and 4A). When $XPR1$ gene expression in DU145 is reduced by siRNA, the efficiency of XMRV entry into these cells is correspondingly reduced (Fig. 5). Taken together, these data suggest that a change in $XPR1$ gene regulation might have occurred either during the transformation process or in the process of generating the prostate cancer cell lines that would enhance XMRV infection. Further studies are required to investigate this phenomenon and to determine whether $XPR1$ gene regulation in different cell types of the prostate gland (PrEC and PrSC) that may consist of fibroblast cells, as well as other cell types in the stroma (12). Related studies have similarly documented XMRV infection of not only prostatic stromal fibroblasts (PrSC) but also human cells of many different tissue types (11, 37), as well as cells from various animal species (37).

The authors of these studies have proposed that the in vivo susceptibility to XMRV cannot be determined by the Xpr1 receptor expression alone but that the ability of different cell types to either transactivate the XMRV viral promoter or to restrict virus replication define how efficient the virus can spread in certain tissues (11). Recent studies have also shown that XMRV infectivity can be limited by the presence of known host restriction factors to retroviral replication, such as the human APOBEC3 and tetherin, as well as mouse APOBEC3, tetherin, and Fv1 proteins (11, 28). It is noteworthy that a number of human cells, including breast cells have been shown to be susceptible to infection by the mouse mammary tumor virus (MMTV) (13, 14), in spite of the fact that the human orthologue of the murine transferrin receptor 1 (Trf1), which is the known cellular receptor for MMTV, is not able to support viral entry in human cells (32).

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The susceptibility of the telomerase-immortalized epithelial cell line (Ep156T) to XMRV infection appears to be markedly increased upon Xpr1 overexpression (Fig. 4C), demonstrating an important role of Xpr1 in determining the XMRV infectivity. In contrast, the smooth muscle cells (Pm151T) did not appear to support any appreciable increase in XMRV infec-
tivity, even in the setting of Xpr1 overexpression via transfection (Fig. 4D). Since RNase L is required for a complete IFN antiviral response against XMRV (7), we determined whether RNase L might also affect the level of XMRV replication in cell culture. The RNase L “Q” variant allele (R462Q) has been shown to significantly reduce the catalytic activity of the RNase L enzyme (5, 44) and to be associated with XMRV infection in prostate cancer patients. Prostate cancer patients who are homozygous (QQ) for this variant allele of RNASEL show 40% association with XMRV infection compared to 1.5% among those who are either heterozygous (RQ) or wild-type (RR) RNASEL carriers (41). On the contrary, Schlaberg et al. recently analyzed 334 consecutive prostate resection specimens collected from a single institution, which show XMRV infection in 6% of the samples by RT-PCR and in 23% of the samples by IHC (34) but show no association of XMRV infection of the tissues with the RNase L “Q” variant allele (R462Q) (34). Specifically, these researchers did not find any difference in RNASEL allelic distribution between XMRV PCR-positive (50% RR, 43% RQ, and 7% QQ) and PCR-negative (42.7% RR, 47.4% RQ, and 10% QQ) cases, suggesting that more men are probably at risk for XMRV infection than have previously been predicted (34). This and other studies (9, 22, 34, 43), which did not find an association of RNASEL and prostate cancer, suggest that population differences and/or other environmental or genetic factors may impact the development of prostate cancer.

Regardless, we decided to genotype the RNASEL gene in most of the cell lines used in the present study. Genotyping of RNASEL showed that the smooth muscle cell line (Pm151T) was indeed homozygous wild-type (RR) at this locus, which might help explain the relatively low level of XMRV infectivity in these cells, even in the condition of Xpr1 overexpression (Fig. 4D). On the other hand, both the epithelial (Ep156T) and the fibroblast (Pf179T) cells were heterozygous (RQ) for RNASEL, a finding consistent with the fact that these cells were more prone to XMRV infection. Although we cannot formally exclude the possibility that other host-factor(s) may also be involved, our results support a role for XPR1 gene expression and RNase L genotype in determining the cellular susceptibility to XMRV infection in vitro. It is noteworthy that the RNASEL locus is found only 1.6 Mb telomeric to the XPR1 gene within the 1q25 HPC1 prostate cancer locus, and evidence of significant association of RNASEL (4, 5, 30, 31) and of nominal association of the XPR1 gene (3) with human prostate cancer has been reported in cases of familial prostate cancer.

In summary, our data reveal important cellular determinants required for XMRV infection of human prostate cells in vitro, which may provide important insights into the possible role of XMRV as an etiologic agent in human prostate cancer. If it can be proven that XMRV infection is indeed widespread and can cause prostate cancer and/or other human diseases (1, 21, 34, 41), it is important to identify effective antivirals against this virus. Toward this end, recent studies have shown that HIV-1 RT inhibitors, such as azidothymidine (AZT) (33, 35), zidovudine (ZDV), and tenofovir disoproxil fumarate (TDF), as well as the integrase inhibitor raltegravir (35), can inhibit XMRV replication in cell culture to various extents, suggesting that they can be used either individually or as a combination therapy to treat XMRV infection (33, 35).

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