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Mutational analysis and glycosylation sensitivity of restrictive XPR1 gammaretrovirus receptors in six mammalian species

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ABSTRACT

Most viruses infect only a few hosts, but the xenotropic and polytropic mouse leukemia viruses (X/P-MLVs) are broadly infectious in mammalian species. X/P-MLVs use the XPR1 receptor for cell entry, and tropism differences are due to polymorphisms in XPR1 and the viral envelope. To characterize these receptor variants and identify blocks to cross-species transmission, we examined the XPR1 receptors in six mammalian species that restrict different subsets of X/P-MLVs. These restrictive receptors have replacement mutations in regions implicated in receptor function, and some entry restrictions can be relieved by glycosylation inhibitors. Mutation of the cow and hamster XPR1 genes identified a shared, previously unrecognized receptor-critical site. This G/Q503N replacement dramatically improves receptor function. While this substitution introduces an *N*-linked glycosylation site, XPR1 receptors are not glycosylated indicating that this replacement alters the virus-receptor interface independently of glycosylation. Our data also suggest that an unidentified glycosylated cofactor may influence X/P-MLV entry.

1. Introduction

Xenotropic and polytropic mouse leukemia viruses (X/P-MLVs) can be isolated from the inbred strains of laboratory mice and some wild mice. All X/P-MLVs use the phosphate exporter XPR1 as their entry receptor (Battini et al., 1999; Giovannini et al., 2013; Tailor et al., 1999; Yang et al., 1999). Co-evolution of the X/P-MLVs and XPR1 in *Mus musculus* subspecies has produced multiple host range variants of the X/P-MLVs and at least six functionally distinct *Mus* XPR1 variants (reviewed in (Kozak, 2011, 2013)). Analysis of these *Mus* XPR1 variants identified two independent receptor determinants in two of its four putative extracellular loops (ECL3 and ECL4) (Marin et al., 1999). Six residues have been identified in these 2 ECLs that govern receptor function (Yan et al., 2010). In *Mus,* there are additional layers of entry level resistance to X/P-MLVs that result from the expression of host-encoded viral Env glycoproteins that block virus infection (Ruscetti et al., 1981), and from glycosylation-based restriction of virus entry (Miller and Miller, 1992; Yan et al., 2009).

X/P-MLVs are broadly infectious in nonrodent mammals (Oie et al., 1976). Although gammaretroviruses have repeatedly invaded mammalian genomes, and endogenous retroviruses (ERVs) related to rodent gammaretroviruses are found in multiple mammalian species (Hayward et al., 2013; Tarlinton et al., 2006), there is no evidence to date that these transspecies transmissions include any of the XPR1-dependent X/P-MLV gammaretroviruses. Interspecies transmission of X/P-MLVs can potentially be inhibited by post-entry host restriction factors, like APOBEC3 (Groom et al., 2010), as well as by species-specific XPR1 polymorphisms that disallow entry of specific X/P-MLVs (Martin et al., 2013; Yan et al., 2010).

Most nonrodent mammalian XPR1 receptors are either fully permissive or are susceptible to subsets of the X/P-MLVs (Yan et al., 2010). This broad conservation of receptor activity results in part from the fact that the key residue for the ECL3 receptor determinant, K500, is at a splice site and is therefore subject to purifying selection, and because receptor function is tolerant of mutations and deletions in the ECL4 receptor determinant (Lu et al., 2014).

Previous analyses of XPR1 function were focused on sequence differences among the functional receptor variants in *Mus* and on

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interference assays of fully permissive XPR1 receptors in other mammalian species (Liu et al., 2016). Here, we expanded these studies to include a set of XPR1 receptors from six mammalian species (cow, Chinese hamster, Syrian hamster, gerbil, bat, dog). Cells of these species have restrictive entry phenotypes, allowing entry of different X/P-MLV subsets. We analyzed these receptors to gain insight into the receptor usage patterns of different X/P-MLVs, receptor plasticity, and potential blocks to trans-species transmission. Mutational analysis of these receptors identified novel sites involved in receptor function, one of which greatly improves the function of the restrictive receptors of cow and hamster. Although three of the six restrictive receptors are sensitive to glycosylation inhibitors and the mutation producing the most significant improvement of receptor function introduces an *N*-linked glycosylation site, the evidence indicates that this substitution alters the receptor-virus interface without involving glycosylation.

2. Materials and methods

2.1. Viruses

Six X/P-MLVs were obtained from J. Hartley (NIAID, Bethesda, MD) and included two X-MLVs: NZB-9-1 and AKR6; three mink cell focus-inducing (MCF) P-MLVs: Friend MCF (FrMCF), Moloney MCF (MoMCF) (Fischinger et al., 1975) and MCF247 (Hartley et al., 1977); and the wild mouse isolate CasE#1 (Cloyd et al., 1985). XMRV X-MLV (xenotropic murine leukemia virus-related virus) (Dong et al., 2007), was provided by R. Silverman (Cleveland Clinic, Cleveland, OH). CAST-X X-MLV was isolated from a CAST/EiJ mouse (*M. m. castaneus*) (Yan et al., 2007) and Cz524 X/P-MLV was isolated from a CZECHII/EiJ mouse (*M. m. musculus*) (Yan et al., 2009).

2.2. Pseudovirus assays

Viral pseudotypes carrying the LacZ reporter were generated for the various X/P-MLVs by infecting GP2-293 cells transfected with pCL-MFG-LacZ as described previously (Yan et al., 2009). Cells tested for virus susceptibility included gerbil GeLu (CCL-100), dog MDCK (CCL-34), cow MDBK (CCL-22), *Mus dunni* MDTF (Lander and Chattopadhyay, 1984), bat Tb-1-Lu (CCL-88), Syrian hamster BHK (CCL-10), Chinese hamster lines E36 (Gillin et al., 1972) and Lec8 (CRL-1737), and stable transfectants of E36 cells expressing various *Xpr1* species variants and mutants. Transfectants produced using Fugene6 (Promega, Madison, WI) were selected with geneticin (830 μg/ml) (Corning Cellgro, Manassas, VA).

Cells were infected with 10-fold dilutions of LacZ pseudoviruses (Yan et al., 2009) in the presence of 4 μg/ml polybrene (Aldrich, Milwaukee, WI). One day after infection, cells were fixed with 0.4% glutaraldehyde and assayed for β-galactosidase activity using as substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 2 mg/ml; ICN Biomedicals, Aurora, Ohio).

Cells were treated with the mannose-mimetic molecule 2-deoxyglucose (2DG, 10–100 mM) and deoxymannojirimycin (DMM, 100 μg/ml) (SIGMA, La Jolla, CA), inhibitors of N-linked glycosylation (Schwarz et al., 1979). 2DG was added to cultures the day before infection and was not removed when pseudoviruses and polybrene were added.

2.3. Cloning and mutagenesis of mammalian Xpr1 orthologs

Xpr1 cDNAs were PCR amplified from cow (MDBK cells), Syrian hamster (BHK) and Chinese hamster (E36) and cloned into pcDNA3.1/V5-His TOPO (Invitrogen, Carlsbad, CA) as described for other *Xpr1* genes (Yan et al., 2009). The sequenced *Xpr1* clones are named for their respective taxa (Table 1) and are identical to Genbank accession numbers NM_001192883, AF198106, and XM_021227866.

Mutant variants at fifteen sites of the mouse (*MdXpr1*), cow (*BtXpr1*) and hamster (*CgXpr1, MaXpr1*) genes were generated using the primers listed in Table 1. Fourteen substitutions were made with QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using as templates the cow, the two hamster clones and the previously described clone of the permissive mouse variant *MdXpr1* (also termed $Xpr1^{sxv}$ (Yan et al., 2007)), identical to GenBank number AF198105. The double mutant R503N/S505A was produced from the cow *BtXpr1*-R503N mutant by amplifying a segment with a forward primer containing the position 505 mutation (Table 1) and a reverse primer from the vector: 5′GACTCGAGCGGCCGCCACTG. The PCR product was digested with *Pac*I and *Not*I and inserted into the *BtXpr1*-R503N mutant clone. All mutants were confirmed by sequencing.

XPR1 expression was confirmed by western analysis. Proteins were extracted from transfected cells with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). The expression vector used for XPR1 inserts a V5 epitope at the C-terminus; XPR1 expression was detected in western blots using anti-V5 antibody (Invitrogen, Carlsbad, CA) followed by goat anti-mouse IgG conjugated with horse radish peroxidase (HRP) (Invitrogen). The membrane was then stripped and incubated with mouse anti-α-tubulin (Sigma, St. Louis, Mo) and goat anti-mouse IgG conjugated with HRP (Invitrogen).

3. Results

3.1. Characterization of six restrictive XPR1 receptors

Cells of six mammalian species were infected with viruses selected for differences in their ability to use the *Mus Xpr1* receptor variants and for sequence variation in the receptor binding domain of envelope (Fig. 1). The sequence differences among these nine viruses are largely concentrated in the two variable domains, VRA and VRB. VRA has been linked to receptor choice (Battini et al., 1992) and while amino acid replacements and an indel in VRA distinguish P-MLVs from X-MLVs and may explain their tropism differences, the responsible Env determinants have not yet been experimentally identified.

Compared to the permissive *M. dunni* MDTF cell line, these six cells restrict different subsets of XPR1 receptor-dependent X/P-MLVs, and the observed infection patterns confirm that X-MLVs have a broader species host range than P-MLVs (Fig. 2). None of these cells can be efficiently infected by P-MLVs, and many also restrict CasE#1 and Cz524, wild mouse isolates that share serological and host range properties with both X- and P-MLVs of laboratory mice (Cloyd et al., 1985; Yan et al., 2009). Bat, dog, gerbil and cow cells are susceptible to some X-MLVs, but Chinese and Syrian hamster cells are uniquely resistant to all X/P-MLVs, including all X-MLVs (Miller and Miller, 1992; Xu and Eiden, 2011; Yan et al., 2010).

Fig. 3 compares the receptor determining regions of the restrictive XPR1 receptors of these six species with the fully permissive receptors of four species (Yan et al., 2010), and identifies the six sites previously implicated in receptor function through analysis of *Mus* XPR1 variants (Marin et al., 1999; Yan et al., 2009). All ten of these species carry the receptor-critical K500 residue in ECL3, and the pattern of substitutions at the other five receptor-critical sites does not correlate with the restrictions detailed in Fig. 2. There are also no replacement mutations at other sites that are common to all six restrictive receptors, although there are specific substitutions at three positions (434, 442, 503) exclusive to the two X-MLV-resistant hamster cells. While restrictive XPR1 variants in *Mus* are marked by three different ECL4 deletions (Yan et al., 2010), there are no deletions in this set of restrictive receptors.

3.2. Mutational analysis

XPR1 receptor genes from two of the most restrictive cells, cow MDBK and Chinese hamster E36, were cloned for mutational analysis. These two receptors differ from each other and from permissive XPR1s in ECL4, in the receptor determining region in the C-terminal end of

Table 1
Primers used for mutagenesis.

Xpr1		Mutation	5′-3′ sequence
Cell Source	*Xpr1*		
M. dunni (*Mus terricolor*)	*Xpr1*^{scv}, *MdXpr1*	D424E/E425D	TGGGAGGACAGTAAGGGCCTGTTGCC
		N432K	GGGCCTGTTGCCAAAAGACCCACAAGAACCAG
		K442R	CCAGAATTTTGCCACAGGTATTCATATGGTGTGCGAGCC
		N503Q	CAGCACTCACAAAGAACAACAGCACTCTGACACCGTGG
MDBK, cow (*Bos taurus*)	*BtXpr1*	E424D/D425E	GAGCTCAAATGGGACGAGAGTGAGGGCCTGTTGCC
		K432N	GAGGGCCTGTTGCCAAACGATTCACAAGAACCAG
		R503N	CAGCACTCACAAAGAACAAAATCACTCAGACACC
		R503G	CAGCACTCAGAAAGAACAAGGTCACTCAGACACC
		S505A	TTTAATTAATGCTGGGAAATACTCCACGACTTTCTTC
			ACGGTGACATTTGCAGCCCTTTACAGCACTCACAAAG
			AACAAAATCACGCTG
		S584L	CCTCCATGACTTCATTGCCTTATTCTGGGGACATC
E36, Ch.hamster (*Cricetulus giseus*)	*CgXpr1*	L434S	GCCTGTTGCCAAATGATTCACAAGAACCAG
		R442K	CCAGAATTTTGCCACAAGTATACATATGGTGTGCGAGCC
		Q503G	CTCACAAAGAACGGGGTCACTCAGATACCA
		Q503N	CTCACAAAGAACGGAACCACTCAGATACCA
BHK, Syr.hamster (*Mesocricetus auratus*)	*MaXpr1*	R442K	CCAGAATTTTGCCACAAGTATACATACGGTGTGCGAGCC

ECL3, and in the N-terminal end of ECL3, a region implicated in receptor function in birds (Martin et al., 2013) but not previously evaluated in mammalian XPR1 receptors. Expression of the cow, *Bos taurus,* receptor (*BtXpr1*) and the *M. dunni* receptor (*MdXpr1*), in E36 Chinese hamster cells reproduced the infection profiles of the endogenous receptors (Figs. 2, 4A and 4B). Ectopic expression of the Syrian hamster (*Mesocricetus auratus*) and Chinese hamster (*Cricetulus griseus*) XPR1 receptors, *MaXpr1* and *CgXpr1*, into E36 hamster cells does not affect virus susceptibility as shown here and previously (Figs. 2, 4C and 4D) (Van Hoeven and Miller, 2005; Xu and Eiden, 2011).

In comparison to the four fully permissive receptors (Fig. 3), cow BtXPR1 has five differences in ECL3 and ECL4 (E424, D425, K432, R503, S584) and hamster CgXPR1 has seven (N426, S428, L434, R442, Q503, A583, Q585). Only one of these sites (503) is altered in both BtXPR1 and CgXPR1, but with different residues. Another of these substitutions is shared by the restrictive bat XPR1 (K432), one by dog (S584) and two by gerbil (A583, Q585). The two largely nonfunctional Chinese and Syrian hamster receptors differ from each other at six sites but both carry replacement mutations at two sites not shared by the permissive or the other partially restrictive receptors, L/I434 and R442.

To evaluate the effect of these substitutions on receptor function, six residues were replaced in cow BtXPR1, three in Chinese hamster CgXPR1, one in Syrian hamster MaXPR1 and five reciprocal substitutions were made in mouse MdXPR1 (Fig. 2). Four of the mutated receptors did not alter virus susceptibility (D424E/E425D, N432K, K442R in MdXPR1; L434S in CgXPR1) (data not shown), but all other substitutions altered virus infectivity patterns (Fig. 4).

Substitutions at position 503 produced the most dramatically altered receptors (Fig. 4). The greatest improvement in the cow BtXPR1 and Chinese hamster CgXPR1 receptors resulted from the introduction of Asn at this position, producing BtXPR1-R503N and CgXPR1-Q503N. BtXPR1-R503N is a highly efficient receptor for X-MLVs including XMRV, for both wild mouse X/P-MLVs, and also allows infection by P-MLVs, which is especially efficient for FrMCF (Fig. 4A). The hamster CgXPR1-Q503N mutant is also a more efficient receptor for most tested viruses, although XMRV, CasE#1 and the P-MLVs are still restricted to various degrees (Fig. 4D). The reciprocal mutation N503Q, however, had minimal impact on the permissive receptor function of the *M. dunni* XPR1 except for reduced infection by FrMCF (Fig. 4B). Introduction of Gly at 503 had very different consequences for the restrictive cow and hamster XPR1 genes; cow BtXPR1-R503G is an improved receptor for all X-MLVs and for FrMCF, but not for other P-MLVs and for Cz524, while hamster CgXPR1-Q503G showed no significant differences compared to the resistant phenotype of CgXPR1 (Fig. 4A, D). This restrictive CgXPR1-Q503G receptor has an ECL3 C-terminus that is identical to that of the fully permissive mink, human and macaque receptors (Fig. 3); while previous studies mapped one of the two independent receptor determinants in mouse XPR1 to the ECL3 C-terminus (Marin et al., 1999), this segment in the three permissive species differs from that of the mouse at three sites and has not been separately evaluated for receptor determinants. Furthermore, the three permissive receptors also differ from that of the hamster at ten additional sites in ECL3 and ECL4 suggesting that while residues at 503 have an important role in receptor function, this role can be mitigated by other local sequence differences.

Several substitutions in the N-terminal end of ECL3 affected virus

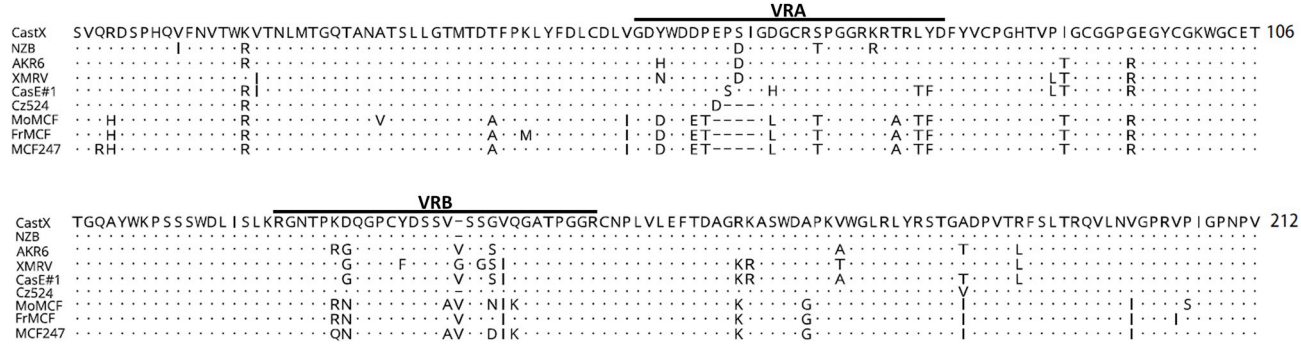

Fig. 1. Alignment of the receptor binding domains in the envelope of six X/P-MLVs that differ in their usage of XPR1 receptor variants. Red bars identify variable domains VRA and VRB. -, deleted residue; ., identical residue.

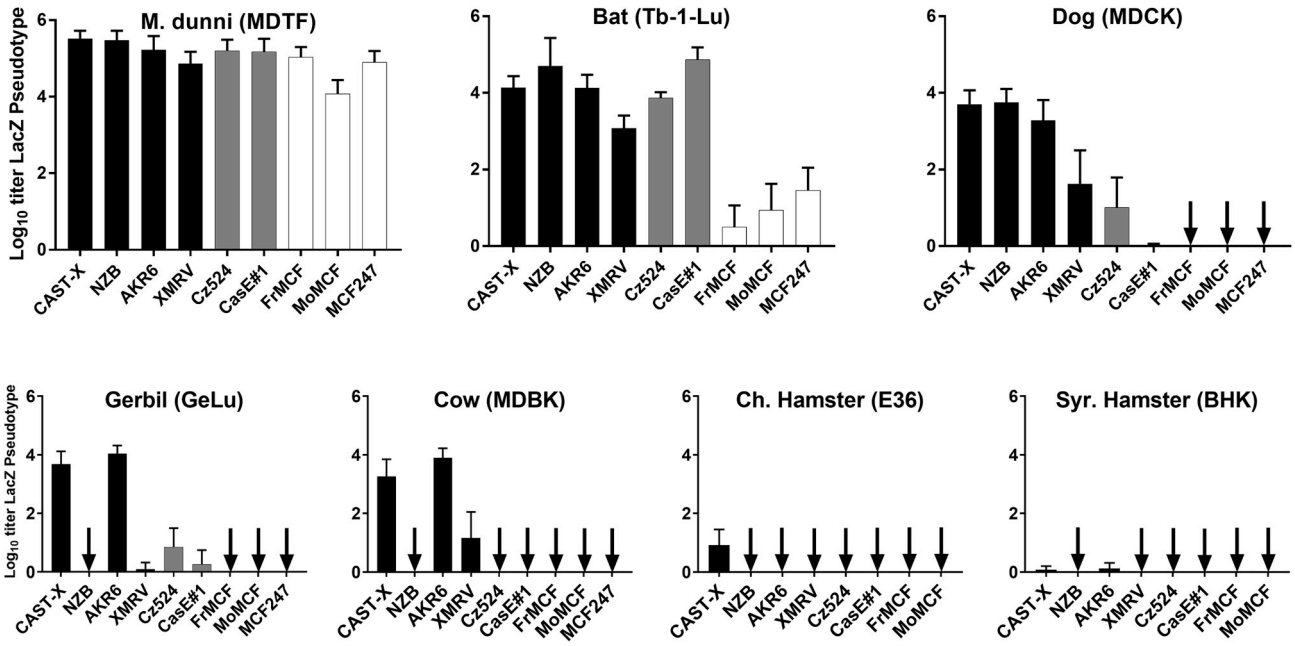

Fig. 2. Virus susceptibility profiles for cells of six species that restrict some or all X/P-MLVs and for the fully permissive MDTF *Mus dunni* cell line. Cells were infected with LacZ pseudoviruses carrying the indicated envelope glycoproteins. Black bars represent the four X-MLVs, the grey bars are the two wild mouse viruses and white bars represent three P-MLVs. Black arrows indicate undetectable infection. Titers were based on 2–8 experiments.

entry. BtXPR1 with the double replacement E424D/D425E showed reduced sensitivity to CAST-X and AKR6, but the reciprocal double replacement in the mouse MdXPR1 had no effect (not shown). Although no receptor changes were observed for MdXPR1-N432K, BtXPR1-K432N was a less efficient receptor than the BtXPR1 wild type (Fig. 4A). MdXPR1-K442R did not alter function, but R442K improved susceptibility to X-MLVs in both hamster XPR1s (Fig. 4C and D) suggesting that the impact of residue changes at these sites is likely dependent on the surrounding sequence.

One ECL4 substitution, S584L, was introduced into BtXPR1 (Fig. 4A). This mutant showed enhanced susceptibility to two X-MLVs, but affected none of the other MLVs.

These results suggest that replacements at position 503 in the C-terminus of ECL3 can substantially improve the function of several restrictive receptors while substitutions at other polymorphic sites have, at best, small receptor-specific effects on function. P-MLVs are the most finicky in their receptor usage; infection by P-MLVs was least likely to be affected by the tested mutations, with the exception of FrMCF P-MLV, which was especially sensitive to reciprocal changes at position 503. This is consistent with our previous conclusion that the various P-MLVs differ in their receptor interactions (Yan et al., 2009).

3.3. Receptor sensitivity to glycosylation

Several of the mutations, L434S, N503Q, and R/Q503N, add or remove the consensus motif for N-linked glycosylation, N-X-S/T (X = !P) where Asn is N-terminal to any amino acid but proline, followed by serine or threonine. One of these three substitutions, R/

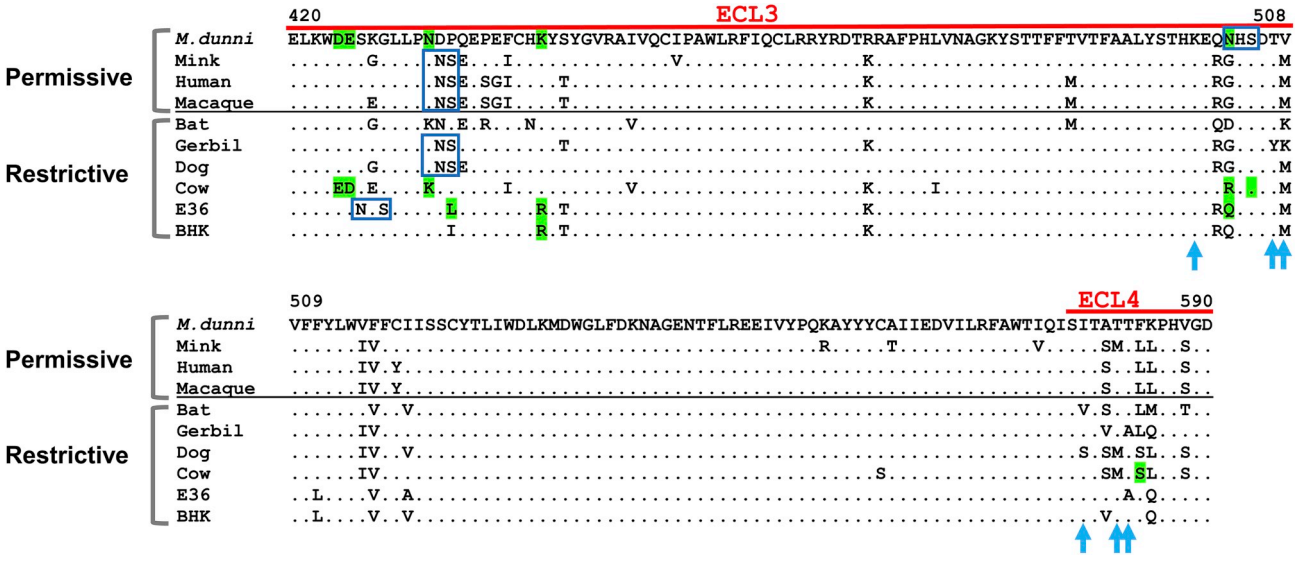

Fig. 3. Alignment of predicted protein sequences of the receptor-determining regions of four permissive and six restrictive XPR1 receptors. ECL3 and ECL4 are marked by red bars, and consensus N-linked glycosylation sites are boxed. Blue arrows identify the sites involved in virus entry for *Mus* XPR1 variants. Mutated residues are highlighted in green.

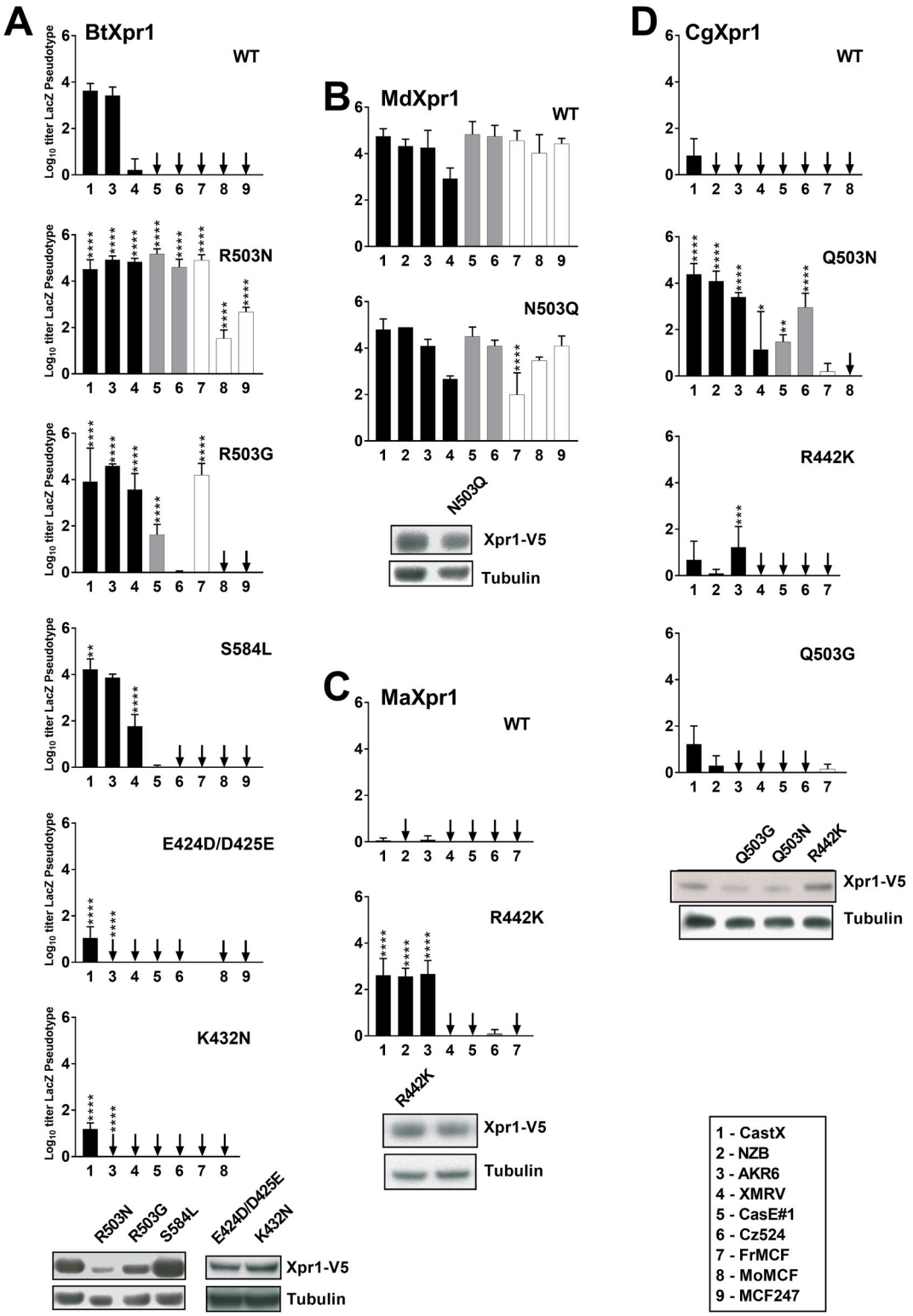

Fig. 4. Susceptibility to LacZ pseudoviruses in E36 hamster cells expressing mutated *Xpr1* receptors from four species. **A)** *BtXpr1*, cow; **B)** *MdXpr1*, *M. dunni*; **C)** *MaXpr1*, Syrian hamster; **D)** *CgXpr1*, Chinese hamster. Black arrows mark undetectable infection. The asterisks identify significant *P* values based on comparisons with the unmutated receptor results at the top of each panel and were determined by the Student's *t*-test (*, $P < 0.1234$; **, $P < 0.0332$; ***, $P < 0.0021$; ****, $P < 0.0001$). Titers were based on 2–8 experiments except for NZB MLV in MdXPR1-N503Q. At the bottom of each panel are western blots showing expression of the V5-tagged XPR1 and tubulin; unlabeled lanes are wild type.

Q503N, produced the most significant improvement in receptor function in cow BtXPR1 and hamster CgXPR1. It has long been recognized that MLV entry can be sensitive to glycosylation inhibition (Wilson and Eiden, 1991). Resistance to some X/P-MLVs in Chinese hamster cells is relieved by inhibitors of glycosylation (Miller and Miller, 1992; Yan et al., 2009) as is resistance to ecotropic MLVs in hamster and rat cells (Tavoloni and Rudenholz, 1997). There are ten putative *N*-linked glycosylation sites in the six restrictive XPR1 receptors and four

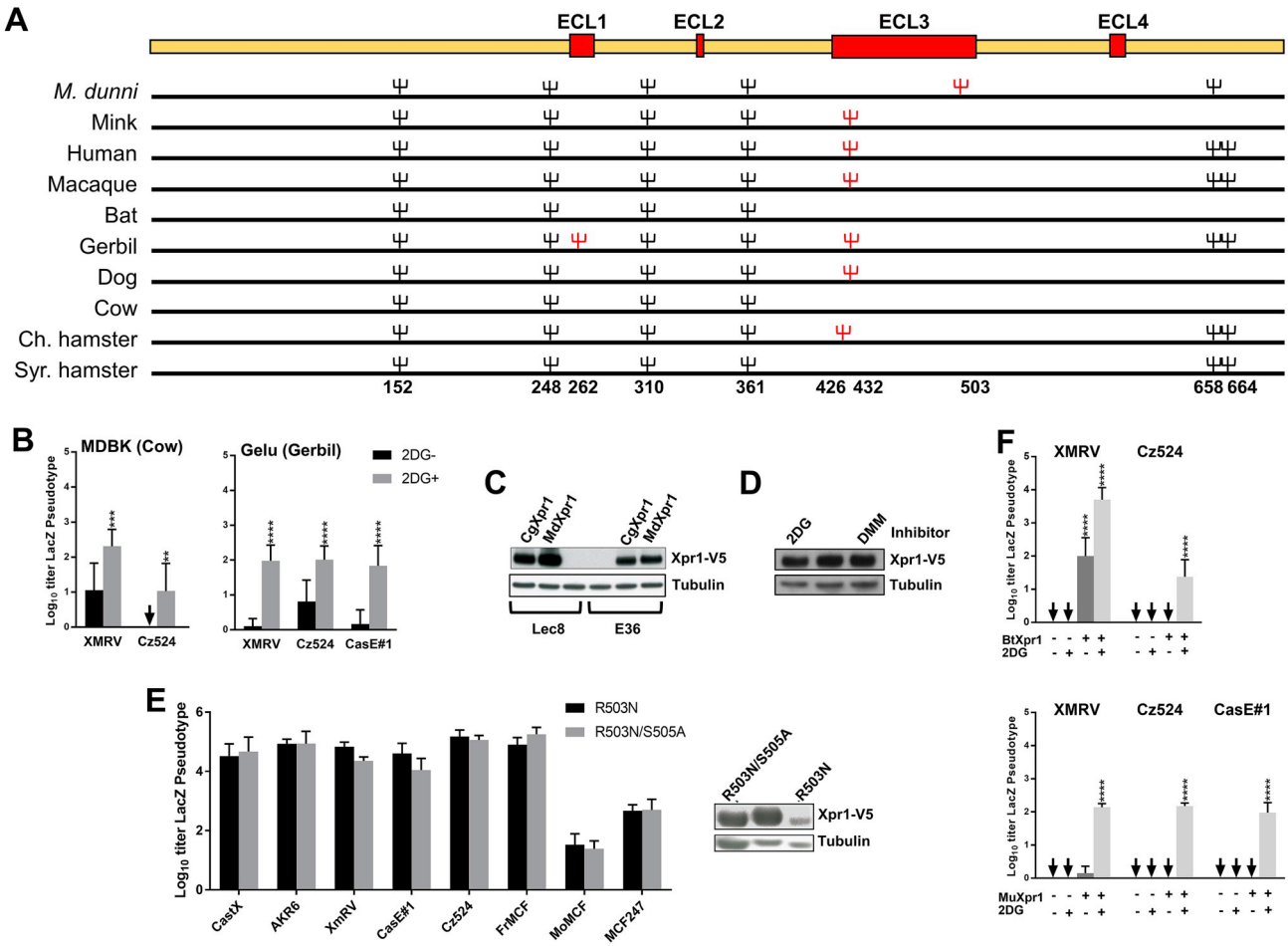

Fig. 5. Effect of glycosylation on virus infectivity. **A)** Number and location of N-linked glycosylation sites in 10 XPR1 receptors. Sites in red are in putative ECLs. The amino acid position of the Asn residues for each *N*-linked site is given at the bottom. **B)** Effect of 2DG-treatment on pseudovirus infections in MDBK and GeLu cells. **C)** Western analysis of two XPR1 genes ectopically expressed in E36 cells and the glycosylation defective hamster Lec8 line. **D**) Western analysis of XPR1 in the presence or absence of glycosylation inhibitors. **E)** Pseudovirus infections in E36 cells expressing BtXPR1-R503N and BrXPR1-R503N/S505A. No statistical differences were observed. Western blots to the right show expression of V5-tagged XPR1 and tubulin. **F)** The effect of 2DG on pseudovirus titers in E36 cells and E36 cells expressing receptors from MDBK (*BtXpr1*) and GeLu (*MuXpr1*). The asterisks identify significant *P* values determined from 2 to 8 independent experiments as determined by the Student's *t*-test (*, $P < 0.1234$; **, $P < 0.0332$; ***, $P < 0.0021$; ****, $P < 0.0001$).

representative permissive receptors (Fig. 5A); only four of these ten sites are shared by all ten species variants. Four of the six polymorphic sites are in the putative ECLs where they could potentially affect virus binding. The XPR1 genes of three species, bat, cow and Syrian hamster, have no N-linked sites in predicted ECLs. These distributions do not correlate with restriction patterns of the different viruses (Fig. 2).

Cells of the six X/P-MLV restrictive species were tested to determine if virus restriction is sensitive to glycosylation inhibition. Treatment with the glycosylation inhibitor, 2-deoxyglucose (2DG) relieved some restrictions in three of these cells but affected different viruses in each of these cells. 2DG-treated MBDK cow cells showed increased susceptibility to XMRV and Cz524, and inhibitor treatment enhanced titers in GeLu gerbil cells for XMRV, Cz524 and CasE#1 (Fig. 5B). We previously showed a 1–2 log increase in infectious titers in 2DG-treated E36 hamster cells for all X-MLVs except for XMRV (Yan et al., 2009).

The presence of a canonical consensus motif does not guarantee that the site will be glycosylated, and in fact, about one-third of such consensus sites are not occupied by glycans (Apweiler et al., 1999). Definitive evidence of glycosylation can be labor intensive, but the presence of glycans can be evidenced by size increases on westerns. Wild type XPR1 receptors with 4, 6, or 7 glycosylation sites show no obvious size differences on westerns, nor do size differences result from the addition or removal of glycosylation sites as shown for mutations R503N, N503Q and Q503N (Fig. 4A, 4B, 4D). Size differences were also not observed for mouse *MdXpr1* and hamster *CgXpr1* expressed in E36 cells compared to hamster Lec8 cells which have a glycosylation defect (Fig. 5C). Finally, treatment of E36 cells expressing cow BtXPR1 with two glycosylation inhibitors produced no change in size (Fig. 5D).

It is possible that only a very small but functional receptor subset is glycosylated. To assess whether the addition of the glycosylation site in cow BtXPR1-Q503N was relevant to the entry phenotype, we introduced a second mutation, S505A, into this construct. S505A removes the glycosylation site, altering NHS to NHA, but does not change the efficiency of infection for any of the MLVs or change the size of the protein (Fig. 5E). These results suggest that XPR1 is not glycosylated, that the 503 substitutions alter the receptor virus binding site independently of XPR1 glycosylation, and that 2DG-treatment affects something other than XPR1 receptor glycosylation.

3.4. Sensitivity of ectopically expressed XPR1 to glycosylation inhibition

A previous study suggested that an unidentified receptor cofactor may contribute to the X-MLV resistance of BHK cells expressing the permissive human XPR1 (Xu and Eiden, 2011). The failure to detect glycans on XPR1 suggests the possibility that glycosylation inhibitors that enhance infectivity may instead target this proposed cofactor. To

determine if differences in species-specific inhibitor sensitivities are receptor-specific or if they are due to a species-specific host cell cofactor that may be glycosylated, we examined the restriction profiles of E36 transfectants carrying cow *BtXpr1* and *MuXpr1* (gerbil, *Meriones unguiculatus*) after exposure to 2DG. Pseudovirus infections were done using viruses that showed 2DG-enhanced infection in MDBK cow cells (XMRV and Cz524) and in GeLu gerbil cells (XMRV, Cz524, CasE#1) (Fig. 5B); none of these viruses show increased infection in 2DG-treated E36 cells (Yan et al., 2009).

E36 transfectants expressing GeLu gerbil XPR1 (*MuXpr1*) were uninfectable by the three test viruses, but 2DG treatment resulted in significant titer increases for all three viruses (Fig. 5F), while not enhancing infection by the P-MLVs (not shown). Untreated E36 cells expressing the cow *BtXpr1* show poor infectivity by XMRV, and resistance to Cz524 (Fig. 5F), the same infection profile observed for MDBK cow cells (Fig. 5B). Treatment of these transfectants with 2DG renders the cells infectible with Cz524 and increases susceptibility to XMRV by almost 100-fold (Fig. 5F); this treatment does not enhance infection by CasE#1 or the P-MLVs (not shown). That 2DG has comparable effects on endogenous and ectopically expressed receptors establishes that the infection block observed for endogenous cow and gerbil receptors is not due to expressed endogenous retroviral glycoproteins that could interfere with receptor binding. These results also suggest that while sensitivity to 2DG is receptor- and virus-determined, multiple lines of evidence indicate that the receptor is not glycosylated, which means that there may be an unknown glycoprotein that facilitates XPR1-mediated entry after 2DG treatment. The data also suggest that this proposed 2DG-sensitive cofactor is present in MDBK cow and GeLu gerbil cells, as well as in E36, where it can be used by the cow and gerbil receptors for infection.

4. Discussion

The majority of mammalian species are susceptible to some or all of the various X/P-MLVs that use the XPR1 receptor. The worldwide distribution of mice harboring X/P-MLVs (Kozak, 2013) has not, however, resulted in documented examples of their cross-species transmission, although there is evidence of multiple jumps involving other rodent gammaretroviruses (Hayward et al., 2013). X-MLVs are subject to restriction by host factors like Fv1 and APOBEC3 (Groom et al., 2010), as well as by entry blocks. Within *Mus* species, the receptor polymorphisms responsible for susceptibility differences likely result from a cyclical host-virus “arms race” in which the development of host survival strategies that block entry alternates with the appearance of X/P-MLV envelope variants. Consistent with this scenario, the rodent XPR1 receptor is under positive selection (Yan et al., 2010), a sign of genetic conflicts. Similarly, X/P-MLV restriction patterns in some avian species have been linked to MLV exposure and the avian XPR1 is also under positive selection (Martin et al., 2013).

While there is no evidence that the extensive sequence variants in nonrodent mammalian receptors evolved in response to virus challenge from X/P-MLVs, their analysis can uncover novel receptor usage patterns that can contribute to our understanding of receptor usage, receptor plasticity and restrictions on cross-species transmission. Our previous analysis of the interference properties of a set of fully permissive XPR1 receptors in four mammalian species showed that these receptor variants are not equivalently recognized by X/P-MLV isolates (Liu et al., 2016). Here we analyzed restrictive receptors from non-*Mus* mammalian species that block different subsets of X/P-MLVs. The limited receptor function in three species, cow, gerbil and Chinese hamster, can be counteracted by glycosylation inhibition, and the function of two restrictive receptors in unrelated species, cow and hamster, is significantly restored by the same substitution, R/Q503N, a change that generates an N-linked glycosylation site.

Glycosylation-mediated restriction that affects cell entry by gammaretroviruses has been attributed to *N*-linked glycans on Env (Knoper et al., 2009), on the receptor (Wang et al., 1996), or on unidentified cofactors (Miller and Miller, 1992). Glycosylated sites are often found at or near the virus binding sites in multiple virus receptors and can serve as recognition epitopes or can block access to nearby receptor binding sites (Tailor et al., 2003). Although the functional alterations at site 503 add or remove an N-linked glycosylation site, we see no evidence that XPR1 is glycosylated at this or any other site. That receptor glycosylation is not involved in altering function is supported by the fact that substitution at this site in BtXPR1 by Gly, R503G, also generates significant improvement of receptor function for several viruses. These Asn and Gly substitutions at 503 may therefore introduce changes in the architecture of the virus-binding interface that affect the efficiency of entry. The 503 residues that generate more functional receptors (Gly and Asn) have smaller side chains than residues in restrictive receptors; these bulky Gln and Arg residues may block access to a nearby binding site centered on K500. Of the sequenced XPR1 genes of mammals and birds tested for function, the only other mammalian XPR1 orthologs with these restrictive residues at 503 are the ungulates goat and buffalo which also have restrictive receptors (Yan et al., 2010). The site N-terminal to 503, 502, also shows significant polymorphism, and the two hamsters and three ungulates are the only species tested for susceptibility that have residues with bulky side chains at both 502 and 503.

2DG shows the same effects on virus susceptibility on endogenous and ectopically expressed cow and gerbil receptors suggesting this phenotype is controlled by the receptor. That the phenotype is replicated in the transfectants also argues against the possibility that the endogenous cow *BtXpr1* and gerbil *MuXpr1* receptors are blocked by expression of an endogenous retrovirus producing an envelope glycoprotein. However, while 2DG, a mannose analog that inhibits protein glycosylation, improves receptor mediated virus entry, several lines of evidence indicate that the receptor protein is not glycosylated: the XPR1 protein size is not altered when glycosylation sites are added or removed by mutation, and glycosylation inhibition does not alter XPR1 size. There are several possible explanations for the effect of 2DG treatment. First, there may be a very small subpopulation of XPR1 that is glycosylated at sites other than 503 but is not detectable in our westerns. Alternatively, an unknown glycoprotein may function as a cofactor in XPR1-mediated entry, and Chinese hamster cells may encode such a factor capable of interacting with the cow and gerbil XPR1 proteins. The existence of such a cofactor was previously suggested in experiments with XMRV-resistant BHK cells that remain resistant even when ectopically expressing the permissive human XPR1. Cell hybrids between these resistant BHK-Xpr1 cells and XMRV-resistant E36 cells are susceptible to XMRV suggesting that E36 supplies a factor needed by the huXPR1 that is absent in BHK (Xu and Eiden, 2011). This could mean that, in some species, exogenous X/P-MLV infections may be restricted at the entry level by a glycosylated cofactor that interferes with the function of some XPR1 receptors.

Conflicts of interest

The authors declare no conflicts of interests.

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