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In Vitro Characterization of a Koala Retrovirus

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Recently, a new endogenous koala gammaretrovirus, designated KoRV, was isolated from koalas. The KoRV genome shares 78% nucleotide identity with another gammaretrovirus, gibbon ape leukemia virus (GALV). KoRV is endogenous in koalas, while GALV is exogenous, suggesting that KoRV predates GALV and that gibbons and koalas acquired the virus at different times from a common source. We have determined that subtle adaptive differences between the KoRV and GALV envelope genes account for differences in their receptor utilization properties. KoRV represents a unique example of a gammaretrovirus whose envelope has evolved to allow for its expanded host range and zoonotic potential.

Members of the family *Retroviridae* are able to reverse transcribe their single-stranded RNA genomes into double-stranded DNA intermediates that are then integrated into the host cell genome as part of the normal virus life cycle. If integration occurs in germ line cells or early-stage embryos, they become endogenous retroviruses (ERV) that can be transmitted vertically via normal Mendelian genetics (4, 7, 9). ERVs have been found in almost all vertebrate species and serve as important indicators of genomic variation (7). Their activation is thought to both protect the host from infection by exogenous viruses and to cause diseases as widespread as immunodeficiencies, cancers, and neurological disorders (4). The recent cloning and characterization of a full-length gammaretrovirus in the genomes of koalas (*Phascolarctus cinereus*) (6) have led us to investigate its relationship with other gammaretroviruses, its host range properties, its virus interference group, and its association with the high incidence of leukemias and lymphomas in its marsupial host (13). The complete sequence of koala retrovirus, herein referred to as KoRV, is most highly related to the exogenous gammaretrovirus gibbon ape leukemia virus (GALV) (6).

KoRV is present and actively expressed within koalas sampled from both captive and free-ranging habitats on mainland Australia. Only recently, a population of KoRV-free koalas was discovered on Kangaroo Island. These animals have been sequestered from the mainland koalas since the 1920s, suggesting that the infection and endogenization of KoRV among mainland koalas are recent events occurring postspeciation (13a).

KoRV occupies a rare status as a gammaretrovirus, since it behaves very much like an exogenous virus and is also present as an ERV; this duality increases the likelihood of self-activation and recombination with exogenous viruses to generate new pathogens with expanded host ranges, as has been documented for feline leukemia viruses (12). To broaden our limited understanding of the relationship between exogenous and endogenous koala retroviruses, we characterized the in vitro host range and receptor utilization properties of the recently identified KoRV and compared these properties to those of the related virus GALV. This comparison will help to establish how a retrovirus was independently mobilized to infect gibbons and koalas and will aid in an understanding of the molecular mechanisms associated with KoRV infection.

A fragment comprising the open reading frame of the KoRV envelope (6) was PCR amplified, sequenced, and subcloned into a mammalian expression vector. Infectivity assays were performed using retroviral vectors encoding the envelopes of KoRV, GALV (SEATO), murine leukemia virus (MLV) 10A1 and 4070A, or the chimeric KGC11D8 (see below), a β-galactosidase genome; either MLV or GALV Gag-Pol and vector titers were determined (14).

Seven amino acid differences (G123E, G225D, Y229H, V230E, P286L, R408H, and S459P) were found between the subcloned envelope sequence and the GenBank sequence (AF151794). KoRV does not require the expression of human PiT1 to infect MDTF cells (in contrast to GALV) and has a lower titer than GALV on MDTF-PiT1 cells (Fig. 1). KoRV enveloped vectors have a more extensive host range than do other gammaretroviruses (Table 1).

The introduction of a C11D8 epitope tag into a specific region of the GALV envelope protein results in a protein capable of being incorporated into infectious GALV particles (3). KoRV and GALV show 72% amino acid identity in their

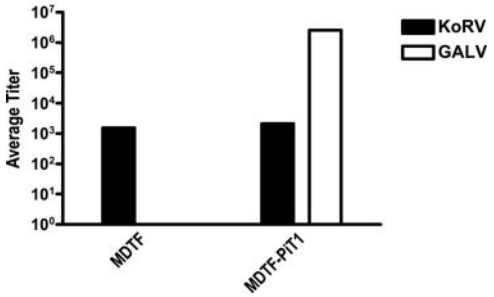

FIG. 1. Comparison of infectivities of GALV and KoRV enveloped retrovirus vectors on MDTF cells and MDTF cells expressing the GALV receptor, PiT1. The titers from at least three independent experiments were averaged and are expressed as mean numbers.

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TABLE 1. In vitro infectivities of KoRV vector pseudotypes[a]

Species	Cell line	Virus titer ($10^3 \pm$ SEM)
Mouse	**MDTF**	1.55 ± 0.13
	SC1	1.01 ± 0.09
	NIH 3T3[#]	0.80 ± 0.04
	MMK	2.51 ± 0.42
Rat	NRK	1.54 ± 0.42
Human	293T	0.97 ± 0.27
	HOS	0.44 ± 0.09
Bovine	**MDBK**[*]	0.97 ± 0.27
Hamster	**BHK**[#*]	2.24 ± 0.34
	E36	1.30 ± 0.26

[a] Virus titers are expressed as mean numbers of blue foci on target cells per milliliter from at least three independent experiments. Cell lines in bold are not infected by GALV. *, cell lines are not infected by A-MLV; #, cell lines are not infected by X-MLVs (11).

envelope sequences, and the majority of the differences reside in variable regions A (VRA) and B (VRB) of the receptor binding domains (Fig. 2a). We constructed a chimeric expression plasmid incorporating VRA and VRB of the KoRV envelope (residues 1 to 248), the C11D8 epitope tag (residues 249 to 266), and the carboxy terminus of the GALV (SEATO) envelope open reading frame. Western blot analysis showed that the C11D8 epitope was detected in viral supernatants (Fig. 2c, lanes 8 and 9) and that the protein was expressed in transfected 293T cells (Fig. 2c, lanes 3 and 4). However, when KGC11D8 chimeric envelope proteins were used in binding assays with receptor-bearing cells, binding was not detected, possibly due to occlusion of the tag upon receptor binding, as seen previously with GALV (3). Alternatively, the KGC11D8 envelope may have binding properties similar to those of the feline leukemia virus FELV-T envelope, which uses PiT1 as a receptor but does not demonstrate efficient PiT1 binding (8). Infections of target cells with KGC11D8 vectors demonstrated

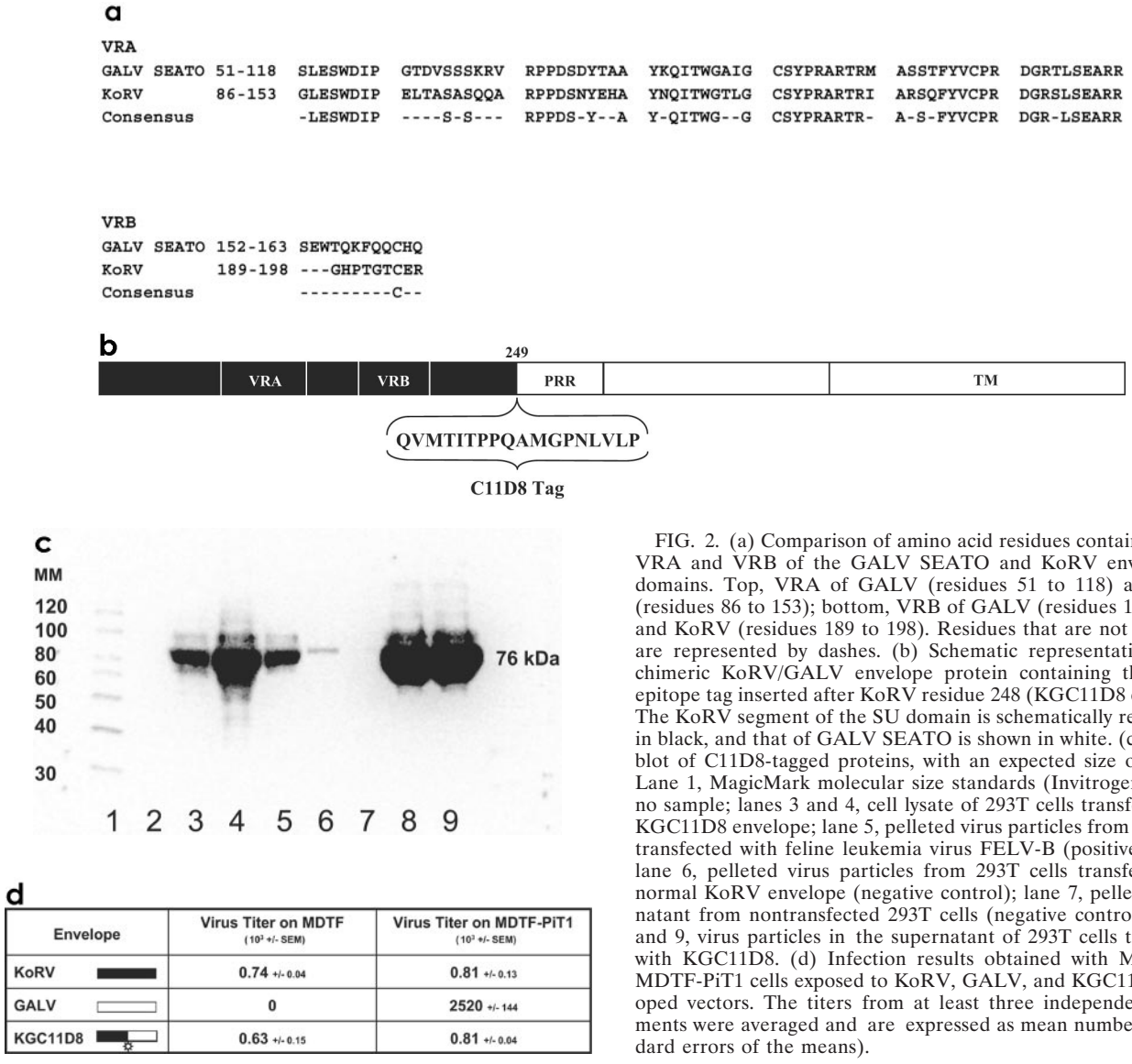

FIG. 2. (a) Comparison of amino acid residues contained within VRA and VRB of the GALV SEATO and KoRV envelope SU domains. Top, VRA of GALV (residues 51 to 118) and KoRV (residues 86 to 153); bottom, VRB of GALV (residues 152 to 163) and KoRV (residues 189 to 198). Residues that are not conserved are represented by dashes. (b) Schematic representation of the chimeric KoRV/GALV envelope protein containing the C11D8 epitope tag inserted after KoRV residue 248 (KGC11D8 envelope). The KoRV segment of the SU domain is schematically represented in black, and that of GALV SEATO is shown in white. (c) Western blot of C11D8-tagged proteins, with an expected size of 76 kDa. Lane 1, MagicMark molecular size standards (Invitrogen); lane 2, no sample; lanes 3 and 4, cell lysate of 293T cells transfected with KGC11D8 envelope; lane 5, pelleted virus particles from 293T cells transfected with feline leukemia virus FELV-B (positive control); lane 6, pelleted virus particles from 293T cells transfected with normal KoRV envelope (negative control); lane 7, pelleted supernatant from nontransfected 293T cells (negative control); lanes 8 and 9, virus particles in the supernatant of 293T cells transfected with KGC11D8. (d) Infection results obtained with MDTF and MDTF-PiT1 cells exposed to KoRV, GALV, and KGC11D8 enveloped vectors. The titers from at least three independent experiments were averaged and are expressed as mean numbers (±standard errors of the means).

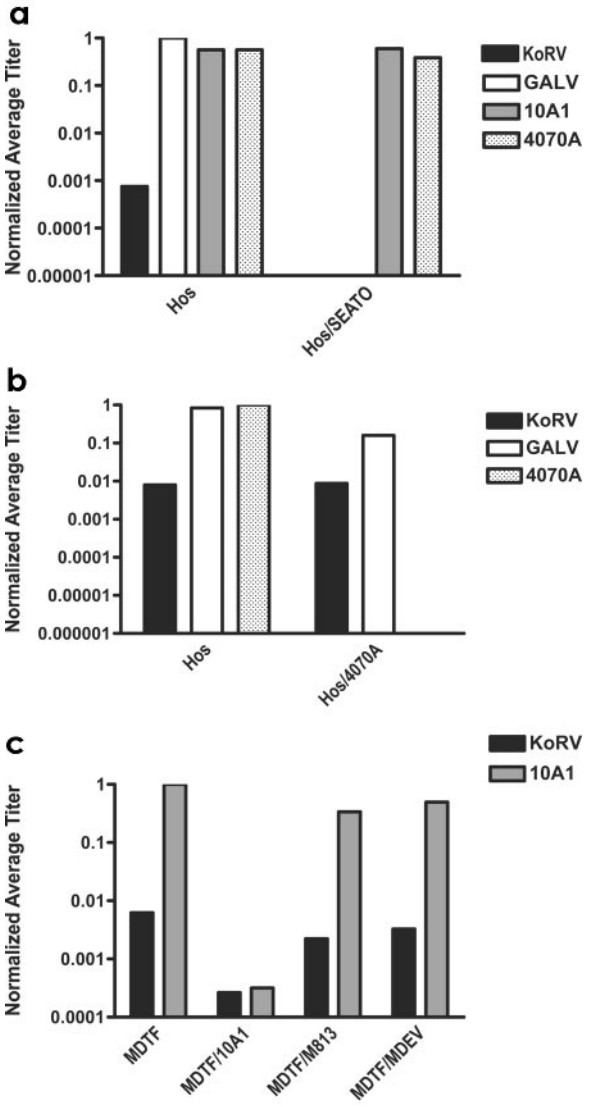

FIG. 3. Results of virus interference studies using superinfection assays. All productively infected cell lines were assayed by their reverse transcriptase activities. (a) HOS cells exposed to KoRV, GALV, and MLV 10A1 and 4070A vectors. In addition, HOS cells were persistently infected with GALV (SEATO) and exposed to the same vectors. Titers are normalized to those of GALV on HOS cells. (b) HOS cells exposed to KoRV, GALV, and MLV 4070A vectors. In addition, HOS cells were infected with MLV 4070A and exposed to the same vectors. Titers are normalized to those of MLV 4070A on HOS cells. (c) MDTF cells exposed to KoRV and 10A1 vectors. In addition, MDTF cells infected with 10A1, M813, or MDEV were exposed to the same vectors. Titers are normalized to those of 10A1 on MDTF cells.

a host range consistent with that of KoRV vectors (Fig. 2d). Hence, the residues that are important in conferring an expanded host range to KGC11D8 reside within the first 248 residues of the KoRV envelope.

Gammaretroviruses are categorized based on the receptors they utilize for infection, although not all viruses in a given receptor class have the same host range (1). GALV utilizes the inorganic phosphate symporter PiT1 as a receptor to infect human cells, and its host range includes cells from species as diverse as felines, canines, bovines, rats, bats, and mink (14). Most murine cells are resistant to GALV. Amphotropic MLV uses a related phosphate transporter, PiT2, and MLV 10A1 can use either PiT1 or PiT2 (11).

Interference assays are based on the observation that a retrovirus will down-regulate the expression of its receptor on target cells so as to block superinfection by a virus that requires the same receptor for entry. Superinfection of HOS cells productively infected with GALV (SEATO) or A-MLV suggested that KoRV uses human PiT1, not PiT2 (Fig. 3a and b). Similarly, superinfection of MDTF cells productively infected with MLV 10A1 (Fig. 3c) demonstrated that MLV 10A1 blocked a KoRV challenge infection, suggesting that like 10A1, KoRV may use the murine ortholog of PiT1 to infect MDTF cells. The data presented in Fig. 3c also demonstrate that KoRV infection of MDTF cells is not mediated by the receptors utilized by MLVs isolated from two different species of Asian feral mice, *Mus cervicolor* (MLV M813) (11) and *Mus dunni* (MDEV) (10).

The recent isolation of a gammaretrovirus from koalas (6) with a nucleotide sequence closely related to that of GALV suggests that the two viruses could be considered conspecific, i.e., derived from a common progenitor (2, 6). Despite their genetic similarities, we have now determined that GALV and KoRV have overlapping but distinct host ranges and receptor utilization properties.

KoRV, an endogenizing genomic relative of the exogenous gammaretrovirus GALV, has a broad in vitro host range differing from that of GALV or any MLV receptor class identified to date. We demonstrated that substitution of the KoRV receptor binding domain is sufficient to confer the infectivity properties of KoRV on a GALV vector (Fig. 2d). Differences in VRA and VRB of the envelope are reflected in key changes in the receptor recognition properties of the KoRV and GALV envelopes. Alterations in envelope composition between KoRV and GALV may account for the ability of these two very similar viruses to infect two vertebrate hosts as diverse as the koala and the gibbon ape. This poses interesting questions on the potential ability of this group of viruses to show further species jumping, which is the subject of ongoing investigation (5).

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