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Author for correspondence:
Jonathan P. Stoye
e-mail: jstoye@nimr.mrc.ac.uk

Apparent effect of rabbit endogenous lentivirus type K acquisition on retrovirus restriction by lagomorph Trim5 α s

Melvyn W. Yap and Jonathan P. Stoye

Division of Virology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

To test the hypothesis that rabbit endogenous lentivirus type K (RELK) could play a role in shaping the evolution of TRIM5 α , the susceptibility of viruses containing the RELK capsid (CA) to TRIM5 restriction was evaluated. RELK CA-containing viruses were susceptible to the TRIM5 α s from Old World monkeys but were unaffected by most ape or New World monkey factors. TRIM5 α s from various lagomorph species were also isolated and tested for anti-retroviral activity. The TRIM5 α s from both cottontail rabbit and pika restrict a range of retroviruses, including HIV-1, HIV-2, FIV, EIAV and N-MLV. TRIM5 α s from the European and cottontail rabbit, which have previously been found to contain RELK, also restricted RELK CA-containing viruses, whereas a weaker restriction was observed with chimeric TRIM5 α containing the B30.2 domain from the pika, which lacks RELK. Taken together, these results could suggest that the pika had not been exposed to exogenous RELK and that endogenized RELK might exert a selective pressure on lagomorph TRIM5 α .

1. Introduction

Retroviruses have a unique replication strategy that involves an obligatory integration step where the viral genome is inserted into that of the host. When this takes place in germ or embryonic cells, the virus can be transmitted vertically to the next generation and is termed an endogenous retrovirus (ERV). ERVs can be fixed in a population, where with time, most of them accumulate deletions, mis-sense and non-sense mutations, leading to a loss of replication competency. While some of the ERVs are co-opted by the host for various functions ranging from development to intrinsic immunity, many are just relics left behind from an ancient infection [1,2]. Like the fossils in paleontology, ERVs provide a means to estimate the age of an infection [3], which could provide insights into the origins of related current exogenous viruses that are in circulation [4,5].

Lentiviruses belong to the genus of retrovirus with members that are a threat to human health, such as HIV-1 and HIV-2. They were thought to be a group of relatively modern viruses until the discovery of the rabbit endogenous lentivirus type K (RELK) [6]. Compared with the primate lentiviruses, RELK has a simpler genome, containing only three accessory genes (*tat*, *rev* and *dUTPase*). However, similar to the primate lentiviruses, their capsid (CA) protein binds cyclophilin A [7]. In addition, viruses containing the RELK CA can also infect non-dividing cells, suggesting that these properties of lentiviruses were acquired a long time ago. Through PCR, RELK sequences were identified in the genomes of several genera of the lagomorph order, including that of the hare, which placed the origins of RELK at more than 12 Mya [8]. Analyses of the PCR products revealed a high degree of homology with the original sequences isolated from the European rabbit. This suggested that invasion of the germline occurred before the divergence of the *Lepus* genus, or that the exogenous virus was widespread among the different lagomorph genera that shared common geographical distributions. However, RELK does not seem to be present in the pika family of lagomorphs [8,9].

Lentiviruses and other retroviruses are susceptible to host restriction factors [10]. One group of restriction factors targets the CA of the virus [1]. These include the murine factor Fv1 [11], as well as TRIM5 α [12] which has been

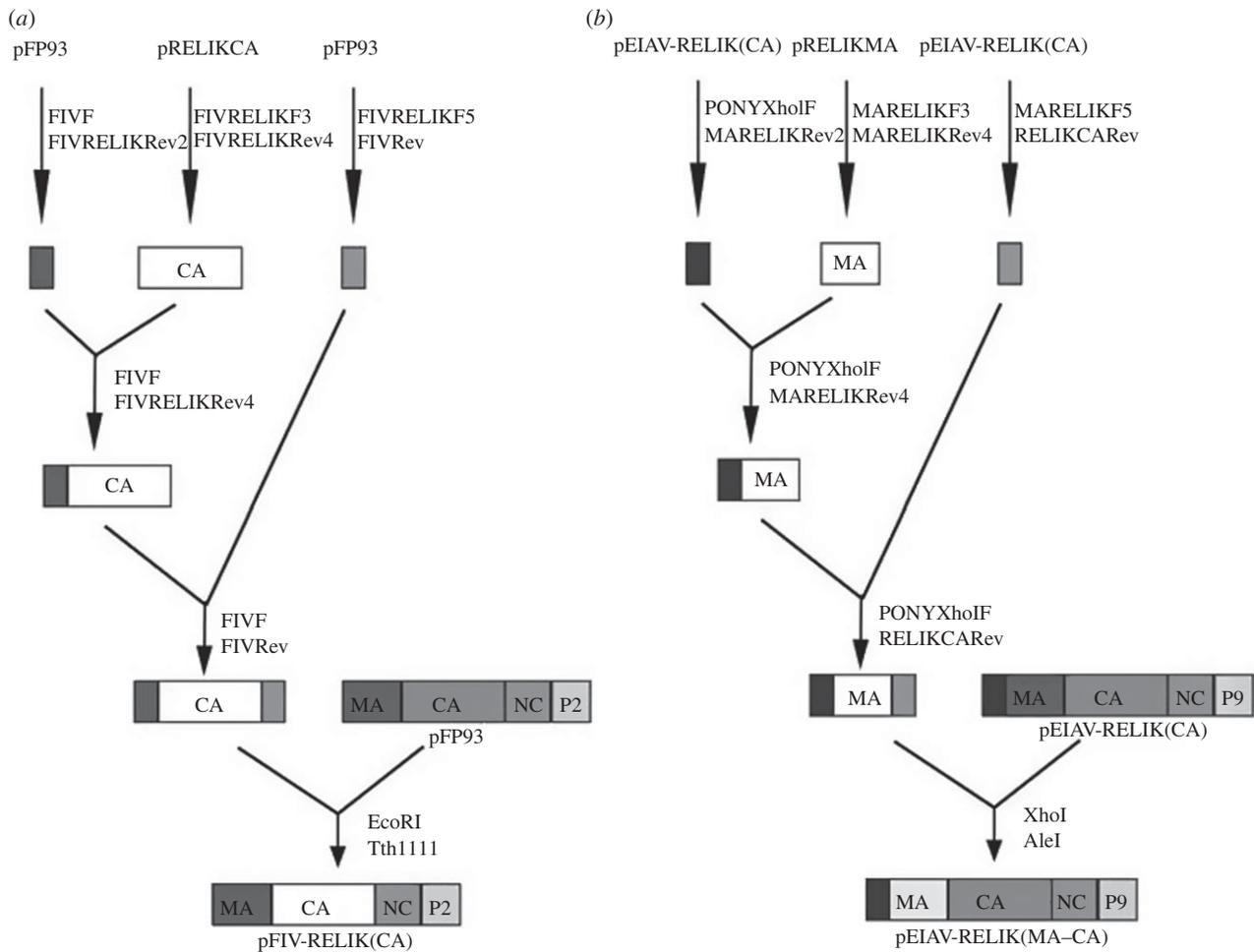


Figure 1. Construction of chimeric *gag* genes. Schematic of chimeric *gag* genes used. (a) pFIV-RELIK(CA); (b) pEIAV-RELIK(MA-CA).

found in many species [13–16]. TRIM5 α belongs to a large family of proteins containing the tripartite ring, B-box and coiled coil (RBCC) motif [17,18]. Some Trim proteins such as TRIM5 α also contain the B30.2 domain at the C-terminus [19,20]. TRIM5 α restricts a range of retroviruses, including lentiviruses [21,22], gammaretroviruses [23], spumaviruses [24] and betaretroviruses [25]. While its precise mechanism of action is still not known, it is clear that multimerization of the molecule that is mediated by the coiled coil is essential [26–29]. The B30.2 domain with its four variable regions [30] has been found to be the major determinant of restriction specificity [31]. It mediates binding to HIV-1 CA [26] and is under strong positive selection [32,33], indicating that it is the part of TRIM5 α that recognizes the virus. Presumably, TRIM5 α evolution is driven by exposure to retroviral CA. However, it remains unclear whether the selective pressure comes from endogenous or exogenous viruses [1].

TRIM5 α orthologues have been isolated from lagomorphs such as the European rabbit and hare [15,34]. They restrict a range of retroviruses, including gammaretroviruses and lentiviruses. In addition, there is strong evidence of positive selection in residues in the B30.2 domain, suggesting that retroviruses could be involved in shaping the evolution of the lagomorph TRIM5 α s [35]. In the light of the prevalence of RELIK sequences in many lagomorph genera, we wondered whether this ancient lentivirus could play a role in the selection of the lagomorph TRIM5 α s. Hence, we set out to investigate the relationship between lagomorph TRIM5 α restriction and viruses containing the RELIK CA. We report here that the TRIM5 α from the cottontail rabbit and pika

can restrict a wide range of retroviruses. In addition, viruses containing the RELIK CA were susceptible to restriction by TRIM5 α s from a number of primates and lagomorphs.

2. Material and methods

(a) DNA constructs

Structures of chimeric *gag* constructs are illustrated in figure 1. The construction of pEIAV-RELIK(CA) has been described previously [7]. pFIV-RELIK(CA) was made by substituting the *EcoRI*/*Tth1111* fragment containing the FIV CA flanked by MA and NC sequences in pFP93 with a corresponding fragment containing the RELIK CA flanked by FIV sequences. The fragment was generated by overlapping PCR using primer pairs FIVF/FIVRELIKRev2, FIVRELIK3/FIVRELIKRev4 and FIVRELIK5/FIVRev in reactions with templates pFP93 [36], pRELIKCA [7] and pFP93, respectively. The first two fragments were joined in a second reaction with primer pair FIVF/FIVRELIKRev4. The resulting product was joined to the third fragment from the first reaction using primer pair FIVF and FIVRev. In order to make EIAV-RELIK(MA-CA), the *XhoI*/*AleI* fragment from pEIAV-RELIK(CA) that contain the EIAV MA was replaced by a fragment containing MA from RELIK (synthesized by Genescript based on the sequence reported by Katzourakis *et al.* [6]). This fragment was generated using overlapping PCR by performing the first reactions with primer pairs PONYXhoIF/MARELIKRev2, MARELIK3/MARELIKRev4 and MARELIK5/RELIKCARev using pEIAV-RELIK(CA) [7], pRELIKMA and pEIAV-RELIK(CA) as templates, respectively. The first two fragments were joined using primer pair PONYXhoIF/MARELIKRev4, and the resulting product was

joined to the third fragment in the first reaction using primer pair PONYXhoIF/RELIKCARev.

TRIM5 α s from the European (*Oryctolagus cuniculus*) and cottontail (*Sylvilagus floridanus*) rabbit were amplified from cDNAs prepared from SIRC and Sf1ep cells, respectively, using the primer pairs RabbitT5F and RabbitT5Rev. mRNAs from these cell lines were isolated using the total mRNA extraction kit from Qiagen and reverse transcribed to cDNA using the first strand synthesis kit from Roche. The TRIM5 α PCR products were cloned into pENTR-D-TOPO (Invitrogen) and sequenced. They were then transferred into pLGatewayIEYFP and pLGatewaySN by an LR recombination reaction using LR Clonase to create the retroviral delivery vectors.

A draft genomic DNA sequence from American pika (*Ochotona princeps*) can be found at http://www.ensembl.org/Ochotona_princeps/Info/Index/. From this sequence, primers targeting sequences in TRIM5 α exon7 and exon8 were designed and used to amplify the B30.2 region from pika genomic DNA (a generous gift of Dennis Lanning, Loyola University). The product was cloned into TopoBlunt and sequenced. Nucleotide and predicted amino acid sequences of the B30.2 coding region were identical to that in the database. To test the restriction properties of pika TRIM5 α , the B30.2 domain was fused to the RBCC of the TRIM5 α from the European rabbit. This was achieved by overlapping PCR using the primer pair TopoRabbitT5F/SIRCPIKARev3 to amplify the sequences encoding the RBCC from the European rabbit TRIM5 α and primer pair SIRCPIKAF3/PIKARev to amplify exon8 from the Pika TRIM5 α encoding the B30.2 domain. The two PCR products were used in a second reaction with primer pair TopoRabbitT5F/PIKARev to generate the chimeric TRIM5 α that was cloned into pENTR/D-Topo before transferring to pLgatewayIRESEYFP and pLgatewaySN by LR recombination. Sequences of these primers are shown in table 1.

(b) Cells and virus production

SIRC (European rabbit), CrFK (cat), D17 (dog), TE671 and 293T (human) cells were maintained in DMEM containing 10% FCS and 1% antibiotics, whereas Sf1ep (cottontail rabbit) cells were maintained in MEM containing 10% FCS, 1% antibiotics and 1% non-essential amino acids. Viruses were made by transfection of 293T cells as described previously. To make the delivery viruses, pVSVG, pHIT60 and either pLgatewayIRESEYFP or pLgatewaySN carrying the various primate and lagomorph TRIM5 α s was used. EIAV and the EIAV-RELIK chimeric viruses were made by transfecting pVSVG, pONY8.4ZCG [37] and either pONY3.1 [37], pEIAV-RELIK(CA) or pEIAV-RELIK(MA-CA), whereas FIV and FIV-RELIK(CA) were made by transfecting pVSVG, pFIVGFP [36] and either pFP93 or pFIV-RELIK(CA), respectively. The virus-containing medium was harvested 48 h post-transfection and concentrated 100-fold by ultracentrifugation for 2 h at 19 500 rpm in a SW28 rotor. Plasmids for producing MLV, HIV-1, HIV-2, SIVmac, PFV, SFV and FFV have been described previously [7,23,24,31,38].

(c) Infectivity and restriction assays

The infectivities of the RELIK chimeric viruses were measured by endpoint titration. Ten-fold dilutions of the virus stocks were made and added to each well of a 12-well plate that had been seeded with 5×10^4 cells per well the day before in the presence of $10 \mu\text{g ml}^{-1}$ of polybrene. The cells were then stained for β -galactosidase 2 days post-transduction, and the number of blue cells counted [39].

To study the restriction of primate TRIM5 α s on EIAV and EIAV-RELIK(CA), vectors carrying the primate TRIM5 α s as well as EYFP were used to deliver the genes into CrFK cells so that more than 80 per cent were transduced. Two days post-transduction, the cells were seeded into 12-well plates and

Table 1. Oligonucleotide sequences.

primer name	primer sequence
FIVF	TTTAAATATGACGGTGTCTACTG
FIVRELIKRev2	ATTCCTGACGTCCATTACTGTTTGAATAGG
FIVRELIK3	AGTAAATGGACGTGAGGAATATGAACCGTTG
FIVRELIKRev4	GCATTTTATAGCTGGTGTGCCAAATTCAC
FIVRELIK5	CAGCACCAGCTATAAAATGCAACTCTTGGC
FIVRev	CCAGTTTCCCGAATCTTTCTATTTT
PONYXhoIF	ATAGGCTAGCCTCGAGGTCGAC
MARELIKRev2	TCCCACCATCTTACTGTCTCTGTGTTC
MARELIK3	GACAGGTAAGATGGGTGGGACGTCCACGTC
MARELIKRev4	ATTCTCAGATCTTTTCTTTTCTTCTTAC
MARELIK5	AGCAAAAGAATCTGAAGAATATCAATCATG
RELIKCARev	TGTTGATATCCACGCTGGTGAAG
RabbitT5F	CACCATGGCTTCAGCAATCTTAGCG
RabbitT5Rev	TCAACAGCTCAACTCGCAGATTG
SIRCPIKARev3	GTCAGCTGGCCCAATAGCGCTGGGCATGTC
SIRCPIKAF3	CGCTATTGGGCCAACGTGACATTGACTCCAAG
PIKARev	CTAGCAAAGCGTCATGGGTCTTG

challenged with different dilutions of the EIAV and EIAV-RELIK(CA) virus. The number of infected cells was determined by staining for β -galactosidase 2 days later.

The restriction phenotype of the lagomorph TRIM5 α s was tested by the two-colour FACS assay as described previously. Briefly, CrFK cells were transduced with delivery vectors carrying TRIM5 α and the EYFP marker. These were challenged with a panel of retroviruses (HIV-1, HIV-2, SIVmac, FIV, EIAV, PFV, SFV, FFV, B-MLV and N-MLV) 2 days post-transduction. The percentages of cells with and without restriction factor that were infected were determined by FACS analyses.

Cat CrFK cells were found to be permissive for the EIAV-RELIK chimeric virus. Single cell clones expressing lagomorph TRIM5 α s were derived from CrFK cells by transduction with limiting dilutions of retroviral vectors carrying both TRIM5 α as well as the G418 resistance marker. The transduced cells were selected on medium containing G418 (1 mg ml^{-1}) for two weeks until visible colonies appeared. Well-separated colonies were picked, expanded and tested for restriction.

(d) Protein analyses

Viruses were pelleted through a 20% sucrose cushion at 100 000g for 3 h at 4°C. The pellets were washed once with PBS and resuspended in SDS loading buffer before separation by SDS-PAGE. The protein bands were then stained with Imperial protein stain (Thermo Scientific).

(e) Nucleotide sequence accession numbers

The gene sequences determined in this study have been submitted to GenBank (accession nos KC425460 and KC425461).

3. Results

(a) Isolation and sequence characterization of TRIM5 α s from lagomorphs

To examine the influence of RELIK on TRIM5 α evolution, we set out to compare the sequences and functional properties of

Table 2. Positively selected residues in lagomorph TRIM5 α s.

codon position ^b	residues present ^c	Bayes factor ^d
328	Q, H, F	329
329	H, G, R	227
330	L, G, N	703
331	S, G, V, N	1112
334	L, G, S	971
341	C, L, F, R	576
342	Q, R, T	152
344	G, E, P	140
346	K, R, T, S	126
389	I, T, Q, M	1534
394	Y, F, P	117
454	K, T, V	133
477	Q, H, T	164
480	K, V, R	119

^aSequences were aligned using ClustalX, and the alignment was used in an analysis for positive selection at <http://www.datamonkey.org/> using the REL method. Residues that are predicted to be positively selected are listed.

^bCodon position based on the sequence of the European rabbit.

^cSingle letter amino acid code.

^dA Bayes factor value between 10 and 100 provides strong evidence for positive selection while those over 100 are decisive.

An alignment of the European and the cottontail rabbit (determined here) with the hare [34], and the pika (inferred from the genomic DNA sequence) TRIM5 α proteins is shown in figure 2*b*. The sequences show marked diversity, particularly in the regions that corresponded to the variable regions (V1–4) previously identified in the primate TRIM5 α s [30,31], with the greatest differences occurring in the V1 region. In this region, the European rabbit and hare sequences are two residues shorter than the pika, whereas the cottontail rabbit alleles lack an additional six residues. In addition, the V3 region of the pika is one residue longer than those of the other species. Differences in the lengths of these regions are frequently observed in primate TRIM5 α [30,40].

A particular feature of genes involved in evolutionary conflicts is a high frequency of non-synonymous nucleotide substitutions. We therefore analysed the sequence data for the presence of positively or negatively selected sites using the DataMonkey webserver. Fourteen positively selected codons were identified (table 2); no negatively selected sites were seen. Thirteen of selected sites mapped within the B30.2 domain with 12 present in the different variable regions, consistent with previous observations on TRIM5 α and confirming that lagomorph TRIM5 α has been subjected to positive selection during the course of evolution [34,35].

(b) Functional characterization of TRIM5 α from lagomorphs

To examine the anti-retroviral activities of the lagomorph TRIM5 α s, CrFK cells were transduced with retroviral vectors encoding the different TRIM5 α proteins, and then challenged with a panel of retroviruses, including gammaretroviruses,

lentiviruses and foamy viruses (figure 3). Individual TRIM5 α s displayed slightly different restriction phenotypes. The foamy viruses PFV and SFV were not restricted by any TRIM5 α , whereas the lentiviruses HIV-1, HIV-2, EIAV, FIV and the gammaretrovirus N-MLV were restricted by all four factors. FFV was restricted only by the TRIM5 α s from the cottontail rabbit, whereas B-MLV and, to a lesser extent, Mo-MLV were restricted by one of the two cottontail alleles. SIVmac was susceptible to that from the pika and one of the cottontail alleles. Because the pika construct possessed an identical RBCC with the European rabbit, we can conclude that the altered specificity for SIVmac is due to a change in the B30.2 domain. However, changes in the overall profile of restriction activity appear relatively small despite significant changes in TRIM5 α sequence.

(c) Chimeric RELIK lentiviral Gag constructs

We next wanted to test whether the sequence changes in TRIM5 α were associated with an alteration in the ability to restrict RELIK. We had previously described the construction of a chimeric lentiviral Gag, EIAV-RELIK(CA), consisting of the RELIK capsid (CA) in a background of EIAV Gag [7] that produced infectious viruses when co-transfected with an EIAV vector and VSV-G into 293T cells. However, the viral titres were significantly lower than the parental EIAV and could only be measured accurately by endpoint dilution after concentration of the viral supernatant. Before embarking on restriction studies with this virus, we wished to investigate whether we could understand the reasons for this low titre. In one approach, the matrix (MA) of EIAV in the chimeric EIAV-RELIK(CA) Gag was replaced with that from RELIK, resulting in EIAV-RELIK(MA–CA). Alternatively, the CA of FIV was replaced with that from RELIK to test the RELIK CA in the background of FIV Gag, yielding FIV-RELIK(CA). The supernatants from 293T cells transfected with VSV-G, an EIAV vector and either EIAV, EIAV-RELIK(CA) or EIAV-RELIK(MA–CA) Gag-pol were pelleted through 20% sucrose cushions and analysed by PAGE (figure 4*a*). The supernatants from 293T cells transfected with VSV-G, an FIV vector, and either FIV or FIV-RELIK(CA) Gag-pol were analysed in a similar way (figure 4*a*). Bands corresponding to the expected sizes for MA, CA and NC were observed in all viral supernatants compared with the mock control. CA from EIAV-RELIK(CA) and EIAV-RELIK(MA–CA) was larger than that of EIAV, whereas the MA band for EIAV-RELIK(MA–CA) was larger than those from EIAV-RELIK(CA) and EIAV. In addition, the CA band for FIV-RELIK(CA) was larger than that of FIV and the same size as those from EIAV-RELIK(CA) and EIAV-RELIK(MA–CA). These results indicated that properly processed viruses were made from all chimeric Gag-pol constructs. Furthermore, the intensities of the bands from different viruses were comparable, suggesting that the viruses with chimeric Gags were assembled, released and processed with similar efficiencies to the parental Gags.

The infectivities of the viruses with chimeric Gags were investigated by titration on a panel of cells of different origins, including human (TE671), canine (D17), feline (CrFK) and rabbit (SIRC) (figure 4*b*). In general, the titres were highest on feline CrFK cells and lowest in rabbit SIRC cells. This was consistent with previous reports of the existence of several blocks to lenti- and retroviral infection in rabbit cells [41]. Hence, CrFK cells were used for all subsequent studies. Introduction of the RELIK MA into the background of EIAV Gag

	European rabbit	cottontail rabbit 1	cottontail rabbit 2	pika
HIV-1	0.10±0.04	0.16±0.06	0.14±0.01	0.10±0.03
HIV-2	0.09±0.05	0.19±0.05	0.12±0.002	0.09±0.01
SIV-mac	0.90±0.15	1.02±0.05	0.16±0.01	0.13±0.06
FIV	0.07±0.04	0.08±0.03	0.10±0.01	0.11±0.06
EIAV	0.07±0.01	0.11±0.001	0.14±0.004	0.06±0.001
PFV	1.01±0.01	1.02±0.03	1.07±0.02	1.03±0.05
SFV	1.01±0.08	0.99±0.03	1.03±0.02	1.01±0.06
FFV	1.01±0.09	0.14±0.05	0.13±0.01	1.00±0.05
N-MLV	0.11±0.02	0.06±0.004	0.14±0.01	0.07±0.002
B-MLV	1.78±0.04	1.84±0.11	0.22±0.03	1.93±0.03
Mo-MLV	1.57±0.01	1.65±0.04	0.38±0.02	1.62±0.07

Figure 3. Retrovirus restriction by lagomorph TRIM5 α s. CrFK cells were transduced with retroviral vectors carrying the different TRIM5 α s as well as the EYFP marker. The transduced cells were challenged 3 days later with a panel of retroviruses carrying the EGFP marker. The ratios of the percentages of infected cells (EGFP positive) containing restriction factor (EYFP positive) to those without restriction factor (EYFP negative) were determined by flow cytometry after a further 2 days. Ratios of less than 0.3 (shaded in black) were taken to represent restriction, whereas ratios greater than 0.7 indicated absence of restriction. Ratios between 0.3 and 0.7 represent partial restriction and are shaded in grey. The numbers represent the average of three experiments.

yielding EIAV-RELK(MA-CA) resulted in a greater than 10-fold decrease in infectivity compared with the chimeric Gag containing only the RELIK CA (EIAV-RELK(CA)) in all cell lines. This suggested that the RELIK MA had a detrimental effect on the infectivity of the chimeric viruses. Alternatively, EIAV Gag could not tolerate the large substitution including both MA and CA. Replacing the CA of FIV with that from RELIK resulted in a large decrease in infectivities in all cell lines. These results showed that the Gag containing RELIK CA that yielded the highest titres was EIAV-RELK(CA). Hence, this was used in all subsequent experiments.

(d) Restriction of RELIK by TRIM5 α from various primates

To examine whether RELIK was susceptible to TRIM5 α restriction, we challenged a panel of permissive CrFK cells transduced with the TRIM5 α from different primates with the EIAV-RELK(CA) virus and EIAV. The results are shown in figure 5. As observed previously, EIAV-RELK(CA) but not EIAV was restricted by the TRIM5CypA from the owl monkey and rhesus macaque [7]. Unlike EIAV, which is restricted more than 10-fold by the four ape TRIM5 α s (orangutan, gorilla, chimpanzee and human), EIAV-RELK(CA) was restricted only by the orangutan TRIM5 α . Both EIAV and EIAV-RELK(CA) were restricted by the three TRIM5 α s from Old World monkeys (sooty mangabey, vervet and rhesus macaque), but not by those from the New World monkeys capuchin, squirrel monkey and common marmoset. EIAV-RELK(CA) is also resistant to the TRIM5 α from the cotton top tamarin (New World monkey), whereas EIAV is modestly restricted (about 10-fold). These results indicated that RELIK can be a target for CA-dependent restriction factors such as TRIM5 α . They also show that the RELIK CA is sufficiently different from that of EIAV to enable it to escape from restriction by the TRIM5 α s from apes and the cotton top tamarin.

(e) Restriction of RELIK by lagomorph TRIM5 α s

The results from the sequence studies provided evidence for the presence of selection forces acting on lagomorph TRIM5 α s, most likely from retroviruses (figure 3 and [15,34]). Because the lagomorph TRIM5 α s were active against

various lentiviruses, it was tempting to speculate that RELIK could be involved in driving the evolution of TRIM5 α in lagomorphs. To test this hypothesis, single cell clones were derived from CrFK cells transduced with the lagomorph TRIM5 α expression constructs by endpoint titration of retroviral vectors carrying the TRIM5 α genes on CrFK cells, followed by G418-selection so that single colonies, which were well separated, could be isolated. This was to ensure that the clones contained a single copy of the lagomorph TRIM5 α gene. Titration of HIV-1 on these clones revealed very similar levels of restriction by TRIM5 (figure 6a) arguing for similar levels of restriction factor expression. The titres of EIAV-RELK(CA) on each clone were then determined by endpoint dilution and compared with the parental CrFK cell line (figure 6b). Compared with the non-transduced control, CrFK cells containing the TRIM5 α from the European rabbit were about 30-fold ($p = 0.0007$) less susceptible to EIAV-RELK(CA), whereas the cells containing TRIM5 α s from the cottontail rabbit and pika showed lower levels of restriction, about ninefold ($p = 0.001$) or fourfold ($p = 0.003$) and fourfold ($p = 0.003$), respectively. These results are consistent with the hypothesis that acquisition of RELIK had provided a selective force leading to the evolution of TRIM5 α with enhanced restrictive activity directed against RELIK. However, we are unable to discount the possibility of other infectious agent(s) exerting the selective pressure on TRIM5 α , resulting in a form which restricted RELIK.

4. Discussion

Consideration of the interaction between the various TRIM5 α s and their targets reveals three riddles that need answering to claim a full understanding of restriction specificity. First, how can Trim5 α from one species recognize and interact with the CA protein of retroviruses from different genera if they share little or no sequence identity? Second, why will Trim5 α from multiple species recognize a given virus even though the interacting residues, thought to lie in the variable regions of the Trim5 α [30,31], differ significantly? Third, given the wide range of possible interactions reflected by questions one and two, why can a single amino acid change, for example the R332P alteration in human TRIM5 α which allows restriction

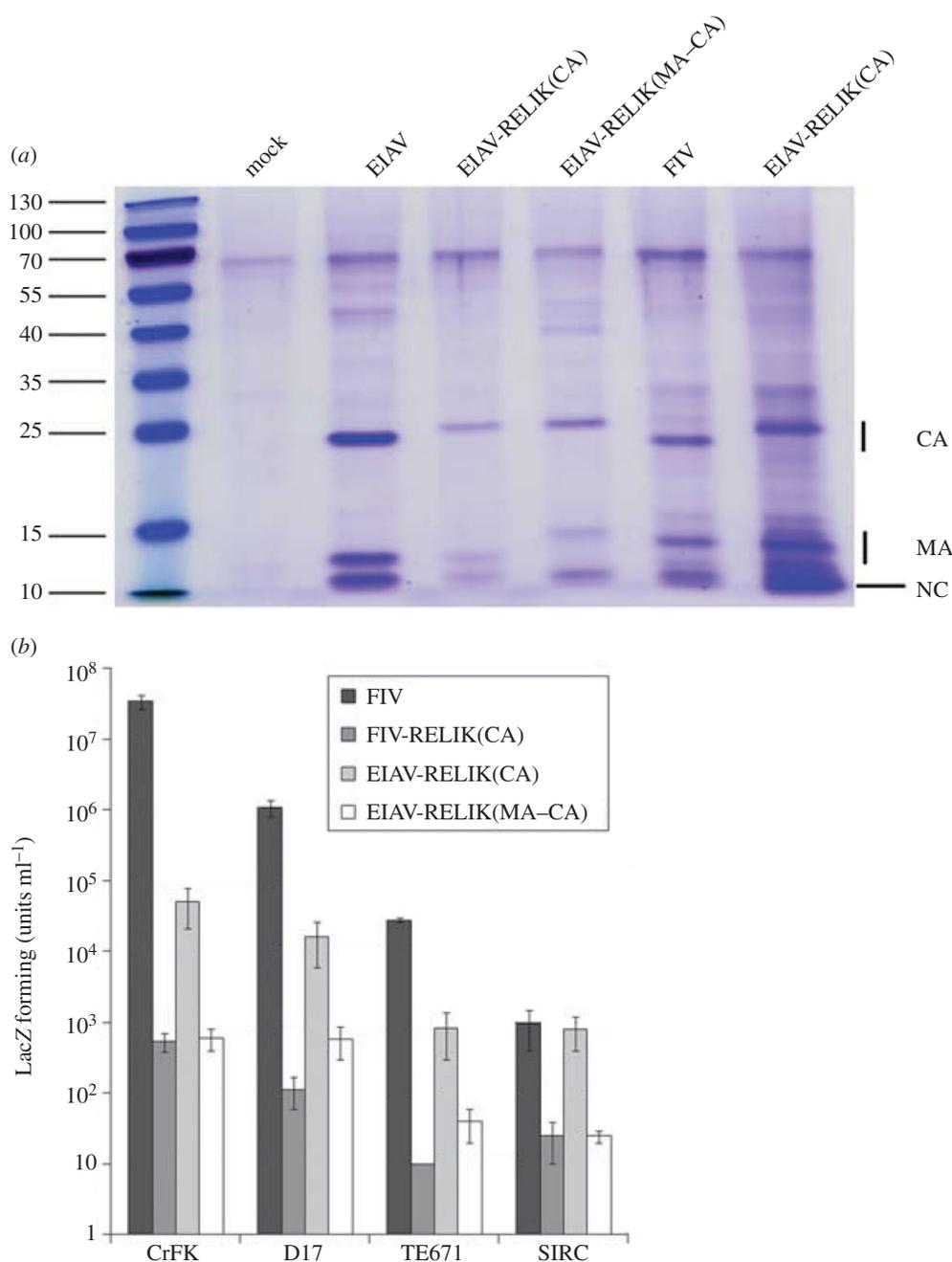


Figure 4. Analysis of chimeric viruses containing the RELIK CA. (a) SDS-PAGE analysis of viral supernatants reveals proper processing of CA. 293T cells were transfected with various constructs containing the RELIK CA. Supernatants were harvested 48 h post-transfection and pelleted through a 20% sucrose cushion before separation on a 12% polyacrylamide gel, followed by staining for protein. Sizes of the markers in kDa are indicated on the left, whereas the viral components are indicated on the right. (b) Titres of viruses containing RELIK CA in various mammalian cell lines. CrFK (feline), D17 (canine), TE671 (human) or SIRC (European rabbit) cells in 12-well plates were transduced with serial dilutions of viruses and stained for LacZ 48 h post-transduction. The number of blue cells was counted in wells containing between five and 500 blue cells and multiplied by the dilution used. Titres were determined as the number of LacZ forming units per ml of virus. Results shown represent an average of three experiments. (Online version in colour.)

of HIV-1 [42] or the R110E change in CA that controls N/B tropism in MLV [43], have such a major effect on restriction?

The properties of the lagomorph Trim5 α s we have examined provide further illustrations of these facets of TRIM5 α restriction. They restrict a variety of viruses including lentiviruses, gammaviruses and foamy viruses (figure 3) with very different CA sequences. Pika and European rabbit recognize many of the same viruses despite having very divergent V1, V2 and V3 regions (figure 2). We know that single amino acid changes in V1 can alter the restriction properties of human TRIM5 α [42,44,45] but a comparison of the Pika (32 amino acids) and European rabbit (30 amino acids) V1 region shows only three amino acid identities, yet both can

restrict HIV-1, HIV2, FIV, EIAV and N-MLV but not PFV, SFV or B-MLV. Interestingly, the two alleles of cottontail rabbit, that are quite closely related (6/64 amino acid differences in V1 plus V2 plus V3), show significant differences in their restriction profiles. In particular, allele-2 was capable of restricting B-MLV, a property of naturally occurring Trim5 alleles not previously observed [31]. We note that multiple alleles of TRIM5 α have previously been reported in European brown hare, Iberian hare and European rabbit [35]. It is therefore very tempting to suggest that this represents a further example of balancing selection among TRIM5 α alleles [46].

It seems likely that the common fold of the CA protein from the Orthoretrovirinae [47] and similar properties

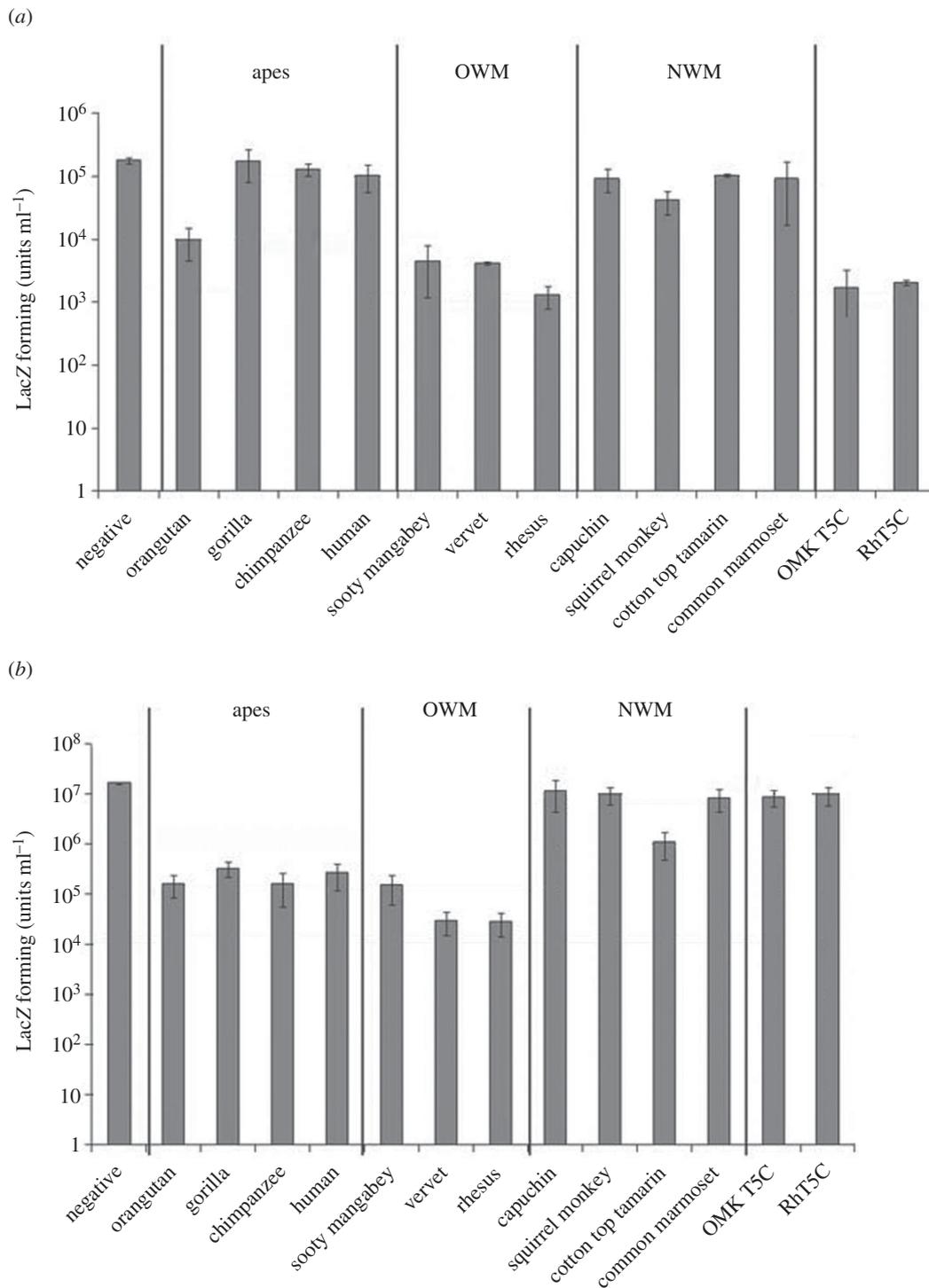


Figure 5. Restriction of EIAV and EIAV-RELIC(CA) by a panel of primate TRIM5 α s. CrFK cells were transduced with retroviral vectors carrying TRIM5 α with the B30.2 domains from the indicated primates before challenging with serial dilutions of (a) EIAV-RELIC(CA) or (b) EIAV. The transduced cells were stained for LacZ 48 h later. The number of blue cells was counted in wells containing between five and 500 blue cells and multiplied by the dilution used. Titres were determined as the number of LacZ forming units per ml of virus. The results are an average of three experiments.

underlying assembly in mature cores [29,47–49], coupled with relatively non-specific interactions between the surfaces of CA and Trim5 [50] will prove important elements in addressing these questions. However, the case of the foamy viruses, which are susceptible to Trim5 α restriction (figure 3 and [24]) but seem to form a rather different structure [51], provides an important complicating factor. Determining the structures of co-crystals between TRIM5 α and a viral target would go a long way to resolving these issues.

TRIM5 α sequences, and therefore their target specificities, have been moulded in large part by a series of positive

selection events [32,33,52]. However, the nature of the viruses driving these changes has been the subject of some debate. It is not clear whether the primary driving force for selection is the need to control ERVs, in which case viruses might be present in the species in which selection took place, or driven by exogenous infection in which case the virus might no longer exist or still be present in the species acting as a source for infection. One case in which a specific group of endogenous viruses has been implicated is the P ϵ ERV1 family that is present in chimpanzees but not in humans [53]. It was argued that the human TRIM5 changed in response to P ϵ ERV exposure

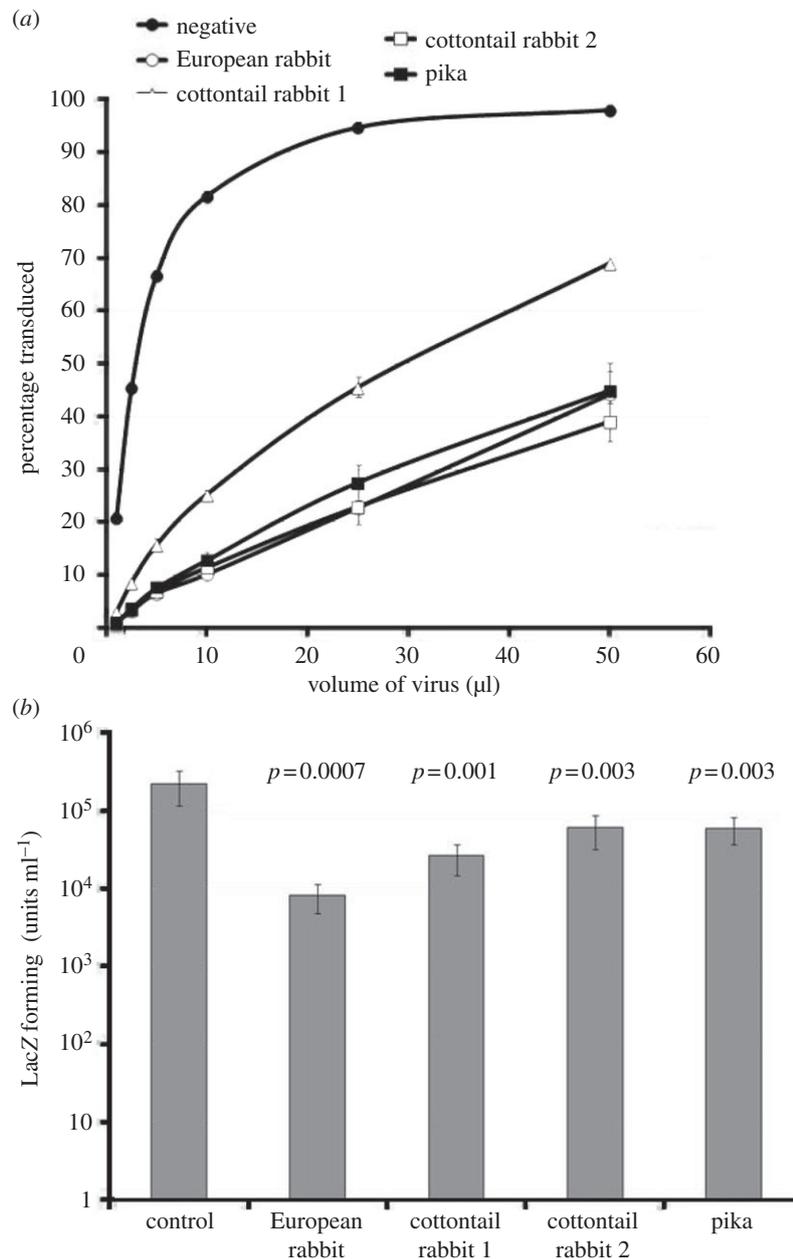


Figure 6. Restriction of EIAV-RELK(CA) by TRIM5 α s from lagomorphs. (a) Single cell clones of CrFK cells established after transduction with retroviral vectors carrying TRIM5 α from the European rabbit, cottontail rabbit or the chimaera containing the B30.2 domain from the pika were treated with increasing volumes (1, 2.5, 5, 10, 25 and 50 μ l) of HIV-1 vector containing the EGFP marker, and the levels of transduction determined by flow cytometry 48 h later. Each data point is an average of two experiments. (b) The single cell clones were challenged with serial dilutions of EIAV-RELK(CA). The transduced cells were stained for LacZ 48 h later. The number of blue cells was counted in wells containing between five and 500 blue cells and multiplied by the dilution factor. Titres were determined as the number of LacZ forming units per ml of virus. The results are an average of three experiments. The *p*-values of Student's *t*-tests between the titres in the control and single cell clones are indicated above the bars.

protecting humans but leaving them susceptible to a later HIV-1 infection. However, others have subsequently questioned this suggestion [54].

The observations that (i) RELIK is present in some lagomorphs but not others [8,34], (ii) positive selection of TRIM5 α was taking place in these animals [34,35], and (iii) our ability to generate an infectious viral vector containing RELIK CA [7] suggested that it should be possible to examine whether the acquisition of RELIK was associated with specific changes in TRIM5 α resulting in RELIK restriction. Before embarking on these experiments, we wanted to see whether it was possible to generate a virus with higher titre than our original construct. We found that EIAV tolerates substitution with the RELIK CA better than FIV, despite RELIK sharing the same late domain with FIV, which is different from that

in EIAV [55]. This could reflect the closer relatedness between EIAV and RELIK as observed from phylogenetic analyses [6,56]. The substitution seemed to compromise infectivity during early events, because processed Gag in the chimeric viruses was produced at levels similar to parental ones (figure 4). In addition, inclusion of the RELIK MA together with CA in the context of the EIAV Gag further reduced the infectivity without affecting viral production. These results suggest that the different viral components continue to have an impact on each other even after virion maturation. In the absence of any evidence of direct interactions between the cleaved viral products, a possible explanation is that they recruit different cellular components that interact with the other viral products. These cellular factors will be more compatible to homologous viral proteins than those from a less related

virus. Examination of these questions might be expected to shed further light on possible roles for Gag products in early, post-entry, events during infection.

Comparison of replication of viral vectors containing the RELIK CA in the presence of TRIM5 α from various lagomorphs revealed a gradient of restriction activity increasing from fourfold for pika, through four- and ninefold for the two cottontail alleles and 30-fold for the European rabbit (figure 6b). The various TRIM5 α proteins show very similar effects on HIV-1 (figures 2 and 6a) arguing against any possible effects of TRIM5 α expression level in the different clones. Such an increase in restriction of RELIK is consistent with a process driven by RELIK exposure. However, the increase is relatively small and these data by themselves do not address the question of whether selection is mediated by endogenous or exogenous viruses.

In the light of the absence of RELIK in the pika genome, one explanation for the low level of restriction of RELIK by the pika TRIM5 α could be that the ancestors of the pika had not been exposed to the virus and, hence, the pika TRIM5 α was not selected for providing resistance to this virus. Alternatively, the low level of restriction could be vestige of a successful protection against RELIK: the pika TRIM5 α could have evolved under selection pressure from other retroviruses following the extinction of RELIK.

We also note that restriction is strongest by the TRIM5 α from the European rabbit, which is the source of the RELIK

sequence used in this study, possibly suggesting that the endogenized virus is most susceptible to the TRIM5 α from its host. One target of TRIM5 α restriction in HIV-1 has been mapped to the cyclophilin A-binding loop between alpha-helices 4 and 5 of the CA [57,58], though other regions may be important [59]. An exchange of the helix 4/5 region between SIV_{mac} and HIV-2 resulted in altered susceptibilities to restriction by the Rhesus TRIM5 α [60]. Although the RELIK sequences seem to be well conserved between the different genera in Leporidae with more than 90% homology, comparison of the cyclophilin A-binding loop between the RELIK CA from the European rabbit and the one sequence of the orthologue found in hares (accession no. FJ493032) revealed four substitutions and two deletions. Because small changes in this region are sufficient to influence restriction, it is tempting to speculate that the RELIK from hares will have different susceptibilities to lagomorph TRIM5 α s. To understand more about the relationship between endogenized lentiviruses and TRIM5 α , it will be necessary to investigate the restriction of RELIK orthologues found in other genera of the Leporidae by the range of lagomorph TRIM5 α s.

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