CHAPTER IV – RETROVIRAL RESTRICTION IN MAMMALS

Background. Retroviruses represent a unique family of viruses because of their ability to insert their genomic DNA into the host chromosome, thus permanently transducing the host with their genetic information. Although this strategy ensures longevity and a constant reproduction for the virus, this transduced information, or the event of transduction itself can be highly pathogenic to the host. It is therefore not surprising that in the course of evolution mammalian cells have been forced to acquire strategies that defend them against retroviral invasion. At the same time, a high morbidity and mortality caused by a virus is not beneficial to the virus itself, as it will result in extinction of the virus from the species. In that case, a virus will undergo selective pressure to acquire the ability of spreading in the host at a rate sufficient to ensure its survival. Thus, selective pressure on both sides will lead to a more benign relationship, and genetic changes in both host and virus that result in lowered pathogenicity will be selected.

Since retroviruses are obligate cellular parasites, in many steps of their life cycle they depend on cellular machinery in order to replicate successfully. Logically, these events of retroviral replication are perfect points of attack for intracellular antiretroviral mechanisms. In fact, many examples of cellular factors inhibitory of retroviral replication have been found in recent years. Thus, the cellular Fv4 gene codes for a defective envelope glycoprotein which blocks Friend virus replication in mice by being incorporated into replication-competent viral particles where it sabotages viral entry into the cell (83, 116). Another example is a recently discovered member of the APOBEC family of cytidine deaminases APOBEC3G. Incorporation of this protein into nascent

HIV-1 particles renders them noninfectious, but only if the viral *vif* gene is disrupted (53, 106). Expression of Vif restores viral infectivity via effects by which Vif counteracts APOBEG3G. This existence of a cellular protein that evolved to inhibit viral replication, and a viral protein that evolved to counteract the inhibitory factor provides an example of a virus-host interplay on the way to a balanced virus-host interaction.

Fv-1: restriction factor that interacts with CA. Inhibitory factors that interact with CA are exemplified by the restriction of certain variants of Moloney Leukemia Virus (MLV) by an activity termed Fv1 in some laboratory mice strains (74, 92). It was originally discovered through an observation that MLV viruses can be classified depending on the mouse strain they replicate in. Based on this criteria, MLV was designated either B-tropic, if it infected the Balb/c mouse strain, or N-tropic, if it infected the NIH/3T3 mice (74).

We now know that this host susceptibility of Murine cells to strains of MLV is mediated by the product of the Fv1 gene. The two major alleles of the Fv1 gene confer resistance to either B-tropic or N-tropic MLV. The product of the $Fv1^n$ allele, originally described in NIH/3T3 mice, confers resistance to B-MLV, while $Fv1^b$ codes for resistance against N-MLV in the Balb/c mouse strain (74, 92). The block has been shown to occur at a step after reverse transcription but prior to integration (61, 93), and the viral determinants have been found to reside in the CA protein of the virus, where a variation of a single amino acid in position 110 in CA determines the N- vs. the B-tropism of the virus (70). The finding that Fv1 restriction activity is overcome by high loads of incoming viral particles has lead to a conclusion that this inhibition is mediated by a

negative saturable factor present in the target cells (17, 33). The Fv1 gene has been isolated by positional cloning and has turned out to code for a Gag-like protein with a 60% similarity to the *gag*-gene of the human endogenous retrovirus HERV-L (16). However, even after the identification of the Fv-1 gene, the detailed mechanism of the Fv-1-mediated restriction is still unknown (9, 16). Presumably, it interacts directly with the CA protein of MLV, since the determinants of viral tropism lie in CA (89). However, such physical interaction has not yet been demonstrated, and the mechanism by which Fv1 blocks viral replication remains a mystery.

Fv1-like restriction activity in mammals. Studies of MLV replication in non-Murine mammalian cells revealed that humans and some Old World primates exhibit a restriction activity against MLV that is reminiscent of the Fv1 restriction in mice. This activity, originally termed Ref1, specifically targets N-MLV but not B-MLV, thus mimicking the phenotype of the Murine $Fv1^b$ allele, and discriminates the two MLV variants based on the residue 110 in MLV CA (14, 121, 122). Restriction to B-MLV has not been observed in any of the cell lines tested.

Mechanistic studies revealed that the Ref1 activity, much like Fv1, can be saturated by flooding cells with large amounts of restricted N-MLV vectors or VLPs, suggesting an existence of a negative, saturable factor present in these cells. However, this factor seemed to interfere with viral replication earlier then Fv1, namely at the level of reverse transcription (14, 121, 122). Intriguingly, humans and primates do not encode the Fv1 gene, nor a gene similar to Fv1 (16).

Existence of an inhibitory factor in humans and primates that so closely resembles

the Murine Fv1 restriction emphasized the role of CA in interacting with host factors during retroviral replication, and pointed out that this kind of CA-specific post-entry restriction might represent a common restriction mechanism among mammals.

Retroviral restriction in monkeys. Yet another CA-specific restriction shed some light on a possible role of CypA in HIV-1 replication, after CypA mission in promoting HIV-1 infectivity had remained a complete mystery for almost a decade.

It has been known for many years that with an exception of the chimpanzees, HIV-1 encounters a strong replication block in non-human primates (7, 58, 59, 72, 107). In fact, efforts to develop an animal experimental model to HIV-1 failed due to this inability of HIV-1 to replicate in monkeys. The block to HIV-1 replication was shown to be specific for HIV-1, since infection by a closely related primate lentivirus SIV is largely permitted in most non-human primates (14, 31, 59, 80). However, models with SIV were not sufficient for vaccine development due to differences of possible viral epitopes, in particular in the envelope glycoproteins, which were found to be important immunogens and candidates for vaccine development (10).

Eventually, chimeric viruses containing pieces of HIV-1 and SIV, designated SHIVs, were made and showed to successfully replicate in rhesus macaques (72, 98, 107). Experiments with SHIVs revealed a rather surprising fact: HIV-1 envelope glycoproteins, which were thought to determine species tropism were able to mediate CD4-dependent entry into macaque cells; instead, the viral tropism determinant turned out to be Gag (107, 108). The determinants of viral susceptibility to this activity were shown to reside in the viral CA protein, as demonstrated by generating chimeric viruses

from pieces from HIV-1 and SIV (36, 86, 87). The activity mediating this block was termed Lv1 (lentivirus susceptibility factor 1) (31).

Much work has been done to characterize this new CA-specific restriction. Studies revealed that it occurs at the time early after entry but prior to reverse transcription (14, 31, 58, 80, 107, 108). To clarify whether the block results from a dominantly acting negative factor in the cell, or whether it is due to inability of HIV-1 to interact with simian factors necessary for early steps of infection, fusions of permissive and restrictive cells were created. These heterokaryons acquired a restrictive phenotype (31), demonstrating that Lv1 represented a dominant inhibitory activity present in the target cells.

One striking difference between replication preferences of HIV-1 and SIV is that SIV CA, unlike that of HIV-1, does not bind CypA (46). Indeed, sequence alignments of HIV-1 and SIV strains show a high variability within their CypA-binding regions (Fig. 4.1-A). Following an intriguing finding that CypA modulates HIV-1 sensitivity to this restriction activity in simian cells, the susceptibility determinant of the virus was narrowed down to the CypA binding site (68, 123). In fact, exchanging the CypA binding site in the CA of a CypA-binding HIV-1 strain against the sequence of a macrophage-tropic, CypA independent virus HIV-1 Ba-L enabled the virus to efficiently infect simian cells (68), while a chimeric SIV encoding HIV-1 CA was devoid of infectivity in rhesus macaque PBMCs (36). Similarly, treatment of target cells with a competitive inhibitor of the CypA-CA interaction Cyclosporin A (CsA), or simply introducing a CA mutation disruptive of CypA binding resulted in relieved restriction in otherwise restrictive monkey cells, increasing HIV-1 titer in cells from owl monkeys, African green monkeys

and rhesus macaques almost to the levels of the unrestricted SIV (11, 12, 123). In cells from owl monkeys, the phenotype was impressively robust: disruption of the CypA-CA interaction rescued the HIV-1 titer by two orders of magnitude on a logarithmic scale (Fig. 4.1-B). This phenotype with respect to CypA binding in primate cells was paradoxical: here, the presence of CypA seemed to inhibit HIV-1 infection by promoting Lv1 restriction, whereas in human cells its binding to CA was required for full infectivity of HIV-1 (Fig. 4.1-B).

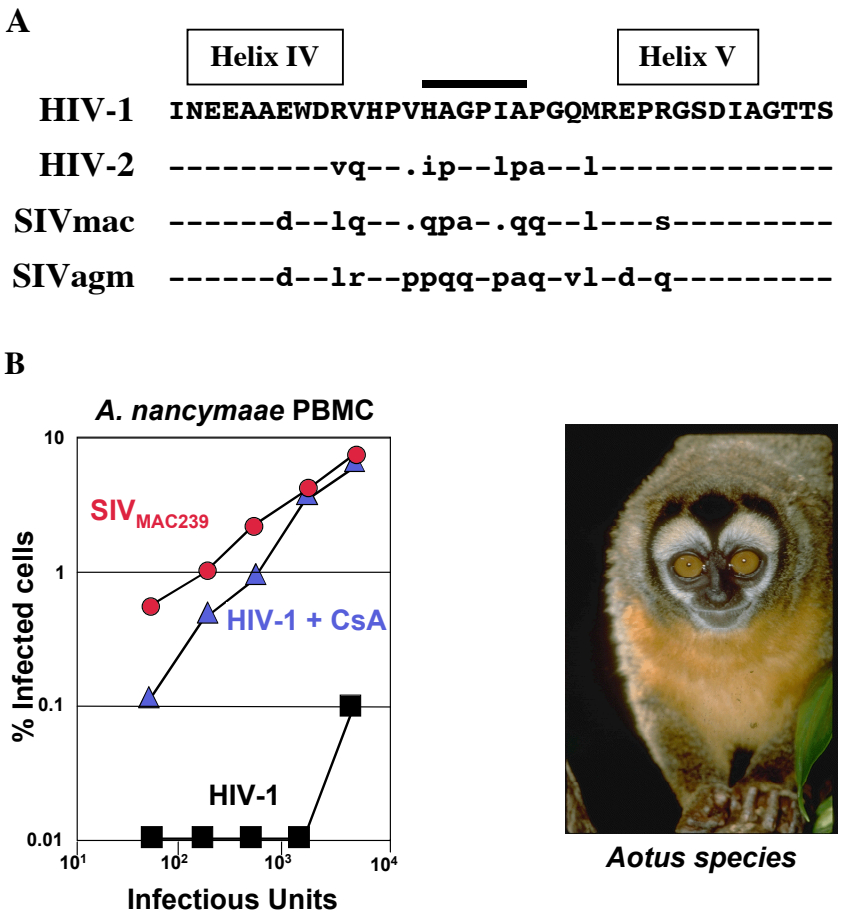


Figure 4.1. HIV-1, but not SIVmac is restricted in non-human primates. (A) Alignment of lentiviral gag sequences. CypA binding site between helices IV and V is indicated. (B) HIV-1 is restricted in owl monkeys (*aotus nancymaae*) and is rescued by CsA almost to the level of the unrestricted SIVmac (103, 123).

Further studies with Lv1 restriction in non-human primates showed that Lv1-mediated restriction is not limited to HIV-1, but characteristic for a broad range of retroviruses. For example, African green monkeys were shown to restrict HIV-2 and SIVmac in addition to HIV-1 (14, 54). Conversely, cells from squirrel monkeys restricted only SIVmac and SIVagm, but not HIV-1 (87). Also, African green monkeys and rhesus macaques were shown to exhibit Ref1 activity in addition to Lv1 activity (15, 121).

Just like Fv1 and Ref1 restrictions, the Lv1 factor can be saturated by flooding the cell with large amounts of HIV-1 particles. Furthermore, if two viruses are restricted by Lv1 in the same species, loading cells with one restricted virus can abrogate restriction of the other virus: for example, SIVmac can abrogate restriction to HIV-1 in African green monkey cells (14, 54). Interestingly, although Ref1 and Lv1 were considered to be two different types of restriction, since they target distantly related retroviruses, a cross-saturation of Lv1 and Ref1 was observed in monkey species that exhibit both types of restriction: infectious titer of N-MLV can be elevated by pre-incubating the cells with HIV-1 (54). This observation raised suspicion that Lv1 and Ref1 activities are involved in the same pathway of restriction, and are possibly even mediated by the same protein.

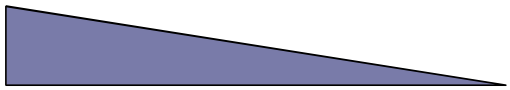
Discovery of TRIM5α. A major breakthrough in the field of HIV-1 restriction in primates was achieved in 2004, when a screen in rhesus monkey fibroblasts identified the host factor mediating Lv1-restriction (114). A cDNA library from rhesus macaques was introduced into permissive human cells, and cells containing a sequence conferring resistance to HIV-1 infection were recovered. The active cDNA was found to encode TRIM5α, a cytoplasmic protein from the family of tripartite motif proteins. Expression of

the rhesus cDNA was sufficient to make human cells resistant to HIV-1. Furthermore, RNAi knockdown of TRIM5 expression in restrictive rhesus macaque cells eliminated the block to HIV-1 infection, providing direct evidence that TRIM5α was required for Lv1 activity (114).

TRIM proteins are named for a cluster of characteristic sequences called the tripartite motif: they contain a RING domain, one or two B-Boxes and a coiled coil domain (95, 114), (Fig. 4.3). The TRIM5 gene gives rise to several isoforms created by differential splicing, with TRIM5α being the only isoform capable of HIV-1 restriction (114). Structurally, this isoform differs from the other TRIM5 proteins in that it contains a C-terminal B30.2/SPRY domain, which also occurs in some other TRIM proteins (95), (Fig. 4.3).

Species-specific recognition. Soon after discovery of TRIM5α in rhesus macaques, TRIM5α orthologues from other species have been isolated and shown to account for restriction activities against a broad panel of retroviruses. As expected, when overexpressed in TRIM5α-negative cell lines, all TRIM5α orthologues restricted the same retroviruses as did the species from which the TRIM5α proteins were isolated. Thus, TRIM5α encoded by African green monkeys and spider monkeys were shown to potently restrict HIV-1 (56, 64, 111), and rhesus, tamarin and squirrel monkey TRIM5α proteins have been found to restrict certain strains of SIV (111), (Fig. 4.2). Confirming the hypothesis, the human TRIM5α was shown to account for Ref1 activity: it was active against N-MLV but not B-MLV and discriminated between these viruses depending of the amino acid residue in position 110 in the CA protein (56, 64, 91, 129), (Fig. 4.2).

In this species-specific recognition of retroviruses, the carboxy-terminal B30.2/SPRY domain was found to play the most important role, as it has been shown to be required for retroviral restriction in most primate species that have been studied (56, 64, 90, 91, 114, 115, 129, 130). The B30.2/SPRY domain was demonstrated to contain determinants for susceptibility and resistance to specific CAs. In fact, sequence differences in this domain among species account for the variation in retroviral restriction specificity among TRIM5 orthologues (101, 111, 114, 130). The most interspecies variations occur within the B30.2/SPRY domain (110). Swapping B30.2/SPRY domains between TRIM5α orthologues confers resistance to viruses that are restricted in the species from which the B30.2/SPRY domain originated (90, 115, 130).



Species	
Rhesus monkey (FRhK4)	SIV_{AGM}, HIV-1, SIV_{mac}, EIAV, N-MLV
AGMtan (COS-1)	HIV-1, N-MLV, SIV_{mac}
AGMpyg (vero)	HIV-1, N-MLV, SIV_{AGM}
AGMtan (CV-1)	N-MLV, HIV-1, SIV_{mac}, EIAV
Human (TE671)	N-MLV, HIV-1, EIAV
Owl monkey (OMK)	HIV-1
Squirrel monkey (pindac)	SIV_{mac}, SIV_{AGM}
Tamarin	SIV_{mac}, SIV_{AGM}, HIV-1, N-MLV
Spider monkey	HIV-1, SIV_{mac}, SIV_{AGM}, N-MLV

Figure 4.2. Restriction of retroviruses by primate TRIM5α proteins. The magnitude of restriction of different retroviruses by each TRIM5a orthologue is indicated. AGM: African green monkey; tan:subspecies *tantalus*; pyg: subspecies *pygerhytrus*. Cellular origin of TRIM5α orthologues is indicated in brackets.

A unique TRIM5 isoform found in owl monkeys accounts for the potent HIV-1 restriction activity in cells from these species. Here, a complete cDNA of CypA has been inserted in frame into intron 7 of all TRIM5 alleles examined (82, 96, 103). In the resulting fusion protein, the B30.2/SPRY domain unique to the alpha isoform has been substituted by CypA. This insertion bears hallmarks of a retrotransposition by LINE1-elements, a poly(A) class of retrotransposons comprising 5% of the human nuclear DNA (40) (Fig. 4.3).

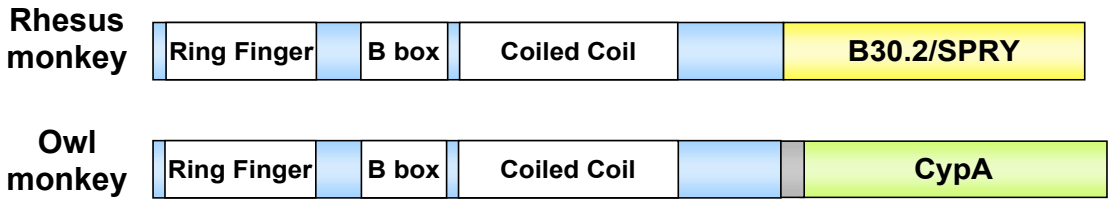


Figure 4.3. Graphic representation of primate TRIM5α structure. In the owl monkey isoform, CypA substitutes the C-terminal B30.2/SPRY domain present in all primate species examined so far.

The discovery of TRIMCyp is particularly intriguing, because it emphasizes the role of CypA in HIV-1 restriction, and the functional interdependence of TRIM5α and CypA. The fact that these two completely unrelated proteins were joined together into one molecule in an event of retrotransposition consolidates suspicion that they both participate in the same process of retroviral restriction. It also explains the unusually robust restriction of HIV-1 in these species: most likely, the CypA domain targets the protein to the CA, thus facilitating restriction by bringing the restriction factor and its target in close proximity.

Monkey TRIM5α and CypA. As described earlier, experiments with non-human

primates suggested that CypA plays an important role in facilitating TRIM5α-mediated restriction, since disruption of CypA-CA interaction results in abrogation of HIV-1 restriction by TRIM5α. However, attempts to demonstrate direct interaction between CypA and TRIM5α utilizing a yeast-two-hybrid system or co-immunoprecipitation were unsuccessful, and the two proteins do not colocalize by fluorescence microscopy (Berthoux, L. and Luban, J., unpublished data). Nevertheless, infectivity studies in Rhesus macaque and African green monkey cells utilizing elimination of TRIM5α and/or CypA expression by RNAi demonstrated that both these proteins influence HIV-1 infectivity via the same mechanism (11). Thus, although physical interaction remains to be demonstrated, it is clear that CypA facilitates TRIM5α-mediated restriction in non-human primates. The mechanism of this interaction remains to be elucidated.

Human TRIM5α and CypA. While CypA's newly assigned role as modulator of HIV-1 restriction in non-human primates has become somewhat clearer, its contribution to HIV-1 infectivity in human cells remained ambiguous. Could it be possible that, similar to monkeys, CypA regulates CA-specific restriction of HIV-1 in human cells? Indeed, some clues from experiments with human and monkey cell lines pointed in this direction.

One evidence for restriction was provided by saturation experiments with heterogonous virus-like particles (VLPs). The titer of N-MLV was increased by loading cells with large amounts of HIV-1 VLPs, but only if CypA binding to HIV-1 CA was abrogated (123). CypA-bound VLPs had no increasing effect on N-MLV infectivity. Similarly, the titer of HIV-1 could be rescued by N-MLV VLPs, but only if the interaction between CypA and CA was disrupted (123). These results have been

interpreted as demonstrating that TRIM5α_{hu} restricts both N-MLV and CypA-free HIV-1 and that each of these viruses can saturate the restriction factor, thus facilitating infection by the other. CypA requirement for full HIV-1 infectivity in human cells was explained by hypothesizing that CypA binding to CA protects incoming HIV-1 cores from restriction by human TRIM5α. This hypothesis was even stronger supported by the discovery of TRIMCyp, the existence of which suggested that the functions of these two proteins are associated.

One even more convincing demonstration was provided by studies with a human cell line TE671, which exhibits a especially strong restriction to N-MLV. In an attempt to investigate whether restriction of N-MLV and CypA-free HIV-1 are mediated by the same activity, cells were subjected to a selection for loss of N-MLV restriction. If the hypothesis was correct, with the loss of restriction to N-MLV the cells were expected to no longer restrict CypA-free HIV-1. HIV-1 has indeed lost its CypA dependence in the selected clone, hereby suggesting that N-MLV and CypA-free HIV-1 are restricted by the same activity. However, further examination of the clone showed that neither TRIM5α sequence, nor TRIM5α expression levels were altered ((102); (Sayah, D.M. and Luban, J., unpublished data).

To directly investigate whether HIV-1 is subject to TRIM5α mediated restriction in the absence of CypA, we chose the RNAi approach to downregulate TRIM5α expression. Loss of restriction to CypA-free HIV-1 with elimination of TRIM5α expression would provide direct evidence that CypA-free HIV-1 is subject to restriction by TRIM5α_{hu}.