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The TRIMCyp genotype in four species of macaques in China

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Abstract The tripartite motif protein (TRIM)5α/CypA fusion protein TRIMCyp in Old World monkeys is generally considered unable to restrict HIV-1 replication. Monkeys with TRIMCyp can serve as a unique animal model for studies of HIV-1 infection. The present study investigated the distribution and expression status of TRIMCyp in four species of macaques originating from China and its borderlands: pigtail macaques (*Macaca nemestrina*), rhesus macaques (*Macaca mulatta*), long-tailed macaques (*Macaca fascicularis*), and Tibetan macaques (*Macaca thibetana*). The results revealed that the frequencies of the TRIMCyp genotype were significantly different among different species and even within different populations of the same species. Interestingly, the TRIMCyp genotype was more prevalent among macaques originating from Yunnan and surrounding regions than those from other regions of China. Importantly, TRIMCyp individuals were first identified in Chinese *M. mulatta* originating from Yunnan, although multiple earlier studies failed to find *CypA* retrotransposition in this subspecies. Furthermore, TRIMe7-CypA, one of the splicing isoforms of the TRIMCyp transcript was expressed in *M. nemestrina* and *M. mulatta* but not *M. fascicularis*. The intra- and interspecies polymorphisms in the deduced TRIMCyp amino acid sequences of these macaques were also analyzed. Taken together, the data in this study provide important information about the genomic background of TRIMCyp among major species of Chinese macaques.

Keywords HIV-1 · Macaques · Polymorphism · Splicing isoforms · TRIMCyp

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Introduction

Several host restriction factors that block HIV-1 replication have been identified, including tripartite motif (TRIM)5α protein (Stremlau et al. 2004), apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G (APOBEC3G) protein (Sheehy et al. 2002), bone marrow stromal cell antigen 2/tetherin (Neil et al. 2008; Van Damme et al. 2008), and the recently reported SAM domain HD domain-containing protein 1 (SAMHD1) (Hrecka et al. 2011; Laguette et al. 2011). The primate TRIM5α was first identified in rhesus macaques (*Macaca mulatta*), a species of Old World monkey, in 2004 (Stremlau et al. 2004).

Additionally, the TRIM5α-cyclophilin A (CypA) fusion protein TRIMCyp, which is encoded by a *TRIM5* gene with a retrotransposed *cypA* pseudogene at intron 7 and can potently restrict HIV-1 replication, was identified in New World owl monkeys (*Aotus trivirgatus*) (Sayah et al. 2004). Subsequently, several research groups confirmed that TRIMCyp was also expressed in pigtail macaques

(*Macaca nemestrina*), *M. mulatta* and long-tailed macaques (*Macaca fascicularis*), which were three species of Old World monkeys (Brennan et al. 2008; Liao et al. 2007; Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008). However, unlike the TRIMCyp in *A. trivirgatus*, the TRIMCyp expressed in most of the Old World monkeys is unable to restrict HIV-1 replication and therefore may be an important target for studies on HIV-1 infection and restriction.

Interestingly, *M. nemestrina* were confirmed to be infected in vitro and in vivo by a modified recombinant HIV-1, in which the viral *vif* gene was replaced by a corresponding gene from $SIV_{mne}027$, $SIV_{mac}239$ or HIV-2 (Hatziioannou et al. 2009; Thippeshappa et al. 2011). It has been speculated that other Old World monkeys expressing TRIMCyp might also be susceptible to HIV-1 infection. Because *M. mulatta* and *M. fascicularis* are the most commonly used nonhuman primates (NHP) models for AIDS studies, TRIMCyp-expressing individuals of these two species of macaques have drawn extensive attention for their potential applications as animal models for direct infection with HIV/SIV chimera viruses (HSIV), in which the HIV-1 genome composition could exceed 95 %. Multiple publications have reported the frequency of the TRIMCyp genotype in *M. fascicularis* and Indian *M. mulatta* (Brennan et al. 2008; Dietrich et al. 2011; Saito et al. 2012; Wilson et al. 2008) but failed to identify this genotype in Chinese *M. mulatta* (Wilson et al. 2008). China has significant endogenous populations of macaques, especially *M. mulatta* (Fooden 2000). However, there have been no extensive investigations on the existence or prevalence of the TRIMCyp haplotype in macaques in the country. In the present paper, we report the investigation of the frequency of the TRIMCyp genotype and its expression status in major species of Chinese macaques to facilitate the development of NHP models that are susceptible to HIV-1 infection.

Materials and methods

Primers

Two pairs of primers were designed to detect the *CypA* insertion in the *TRIM5* locus at the DNA and cDNA levels. The primers were designed according to the *TRIM5* sequence located at the chromosome 14 of macaques (GenBank accession No. NC_007871). The positions and sequences of these primers are presented in Fig. 1. The primer sequences were, E6: 5′-CATGACCTTGAAG AAGCC-3′; U3: 5′-TAGCATATCCATCACCTCAAA-3′; E2: 5′-TACCAGCCTGAGAACATACAGC-3′; Ec: 5′-TTATTCGAGTTGTCCACAGTCAG-3′. The primers used for the amplification of macaque mitochondrial DNA were: 5′-GCGCCCACTCAGCCAATTCCTGTTCT-3′ (forward) and 5′-GCGTGATCCATCGAGATGTCTT-3′ (reverse) (Smith and McDonough 2005).

Preparation of DNA and RNA samples

A 100-μl sample of blood (approximately 10^7 PBMCs) from each animal was subjected to genomic DNA extraction using the Qiagen Blood DNA Extraction Kit (Qiagen, Germany) or total RNA extraction using the Axygen RNA Isolation Kit (Axygen, USA) according to the manufacturer's instructions. The cDNA was synthesized using the Invitrogen RT-Kit (Invitrogen, USA).

Macaque species and origins

The *M. nemestrina* (*Macaca leonine*, a regional subspecies of *M. nemestrina*) originally came from wild animal salvage centers of Guangdong Province and were raised at the Guangdong Landau Biotechnology Co. *M. fascicularis* (originating from Indochina) and *M. mulatta* (originating from the Yunnan and Guangxi regions of China were also bred at Landau. *M. mulatta* originating from the Sichuan region and *Macaca thibetana* (Sichuan origin) were bred at the Sichuan Yibin Hengshu Bio-Technology Co. *M. fascicularis*, *M. mulatta*, and *M. thibetana* were F1 descendants

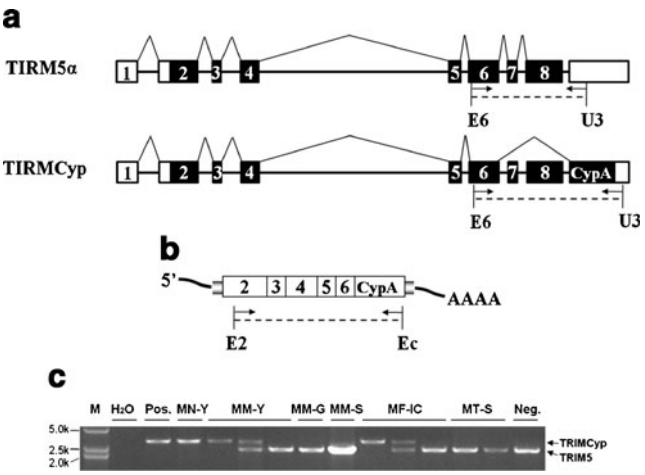

Fig. 1 Detection of TRIMCyp genotypes in macaque species. **a** The positions of primer pairs used to detect TRIMCyp genotypes are presented. The E6/U3 primer pair was screened from a group of primers to identify *CypA* insertion into the *TRIM5* locus. The forward primer E6 was anchored at exon 6, and the reverse primer U3 recognized the 3′-UTR region. **b** The E2/Ec primer pair was designed to amplify TRIMCyp cDNA for the analysis of alternative splicing isoforms. **c** The E6/U3 primer pair, which distinguished TRIMCyp homozygotes and heterozygotes and *TRIM5* homozygotes, was used to investigate the presence of TRIMCyp in *M. nemestrina* (*MN*), *M. mulatta* (*MM*), *M. fascicularis* (*MF*), and *M. thibetana* (*MT*). *M. mulatta* originated from three provinces of China, including Yunnan (*Y*), Guangxi (*G*), and Sichuan (*S*). *M.* fascicularis originated from Indochina (*IC*). *M* molecular markers, *Pos* positive control using *M. nemestrina* chromosomal DNA, *Neg* negative control using randomly scrambled primer pair, H_2O water control

of breading colonies, each of which contains 1 male and 10–15 female monkeys.

PCR amplification

The LA-Taq PCR Kit (Takara, Japan) was used in this study. The program for PCR amplification of genomic DNA was as follows: preheating at 95 °C for 5 min; 30 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 90 s; a final hold at 72 °C for 5 min and storage at 4 °C. RT-PCR was performed by incubation at 37 °C for 1 h; 95 °C for 5 min; 30 cycles of 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 90 s; a final extension at 72 °C for 5 min and storage at 4 °C. The condition for mitochondrial DNA amplification was at 94 °C for 5 min; 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 45 s; and a final hold at 72 °C for 5 min and storage at 4 °C.

DNA cloning and sequencing

The PCR products were purified by agarose gel electrophoresis using the Qiagen DNA Purification Kit (Qiagen) and then subcloned into the pGEM-T vector (Promega, USA) and sequenced by Sangon (Shanghai, China). At least 20 clones for each animal were randomly selected for sequencing. Sequences were analyzed using the Lasergene 7.0 (DNAStar, USA).

Polymorphism analysis

Sequences were assembled using the SeqMan program, and aligned or phylogenetically analyzed using the programs MEGA and Phylip 3.69. A neighbor-joining tree of TRIMCyp and TRIM5α sequences was generated using the MEGA 3.1 program. Additional sequences used in the TRIMCyp alignment included *M. nemestrina* (GenBank Accession No.AY899887.1-AY899893.1) and AT (AY646198.1). The reference sequences used in the phylogenetic analysis of rhesus macaque mitochondrial DNA included Bangladeshi (AB275633–AB275639) and Japanese (AB261566, AB261568, AB261570, AB261572, AB261574, AB261576, and AB261578) *M. mulatta*, Chinese *M. mulatta* originating from regions of Anhui (AF135271), Fujian (AF135272–AF135278), and Yunnan (AF135339–AF135352).

GenBank accession numbers of nucleotide sequences obtained in this study

The GenBank accession numbers for the cDNA sequences of macaque *TRIM5* and TRIMCyp, as well as for the mitochondrial DNA sequences of *M. mulatta* originating from Yunnan obtained in this study are listed in the Table 3 in the Electronic supplementary material.

Results

The existence and frequency of TRIMCyp in major macaques of Chinese origin

To screen for individuals expressing TRIMCyp in major macaques of Chinese origin, a pair of primers was designed according to the consensus sequence of exon 6 (E6) and the 3′-UTR region of the *TRIM5* gene (U3, Fig. 1a) based on sequences registered in GenBank. This pair of primers was expected to amplify a 2.4-kb fragment from the *TRIM5*-bearing chromosome and a 3.0-kb fragment from the *TRIM5* gene with a *CypA* insertion. Both of the *TRIM5* locus alleles in *M. nemestrina* have an insertion of the *CypA* pseudogene; therefore, TRIMCyp was first amplified in 10 *M. nemestrina* as the positive control. As predicted, a single 3.0-kb fragment was amplified using the E6/U3 primer pair in all of the *M. nemestrina* DNA samples. Sequence analysis further confirmed the *CypA* insertion (data not shown). Subsequently, chromosomal DNA samples were collected from 1,040 continental *M. fascicularis* originating from Indochina, 50 *M. thibetana*, and 600 *M. mulatta* and analyzed for *CypA* insertion. The *M. mulatta* originated from Yunnan, Guangxi, and Sichuan provinces of China (200 animals each).

Among all of the species evaluated, *M. fascicularis* displayed a relatively high frequency of TRIMCyp (2.3 % homozygotes and 9.4 % heterozygotes), and no *CypA* insertion was detected in *M. thibetana*. Interestingly, the *CypA* insertion in Chinese *M. mulatta* showed a dramatic geographic distribution. The TRIMCyp haplotype was first identified in Chinese *M. mulatta* originating from Yunnan province, with an allele frequency of 0.5 % homozygotes and 2.5 % heterozygotes within the 200 individuals examined. No individuals with the TRIMCyp haplotype were identified among the 200 *M. mulatta* orienting from Guangxi or the 200 *M. mulatta* from Sichuan (Table 1). The regional difference in the frequency of TRIMCyp alleles was statistically significant between the *M. mulatta* population from Yunnan and those from Guangxi and Sichuan ($P<0.01$). Representative *TRIM5* and TRIMCyp PCR fragments amplified from DNA samples of each macaque species examined are shown in Fig. 1c.

Because reported investigations failed to identify TRIMCyp genotype in Chinese *M. mulatta* and this genotype was detected in *M. mulatta* only from Yunnan but not from other major *M. mulatta* habitats in China in this study, it was important to confirm that these rhesus monkeys were really Chinese species and originating from the region of Yunnan. Mitochondrial DNA samples from three Yunnan *M. mulatta* examined in this study were cloned and sequenced. These sequences were aligned with GenBank-registered mitochondrial sequences from Bangladeshi and Japanese *M. mulatta* and Chinese *M. mulatta* originating from provinces of Fujian, Anhui and Yunnan, and their

Table 1 Allele frequency of TRIMCyp among four species of macaques originating from China and bordering regions

Species Genotype	*Macaca fascicularis*	*Macaca nemestrina*	*Macaca mulatta*	*M. mulatta*	*M. mulatta*	*Macaca thibetana*
Homozygous TRIMCyp	48/2,080 (2.3 %)[a]	20/20 (100 %)	2/400 (0.5 %)	0/400	0/400	0/100
Heterozygous TRIMCyp	196/2,080 (9.4 %)	0/20	10/400* (2.5 %)	0/400	0/400	0/100
Homozygous *TRIM5*	1,640/2,080 (78.8 %)	0/20	378/400 (94.5 %)	400/400 (100 %)	400/400 (100 %)	100/100 (100 %)
Total individuals	1,040	10	200	200	200	50
Regions of origin	Indochina	Yunnan	Yunnan	Guangxi	Sichuan	Sichuan

*$P<0.01$ when compared with *M. mulatta* originating from Guangxi and Sichuan

[a] TRIMCyp allele frequency

phylogenesis were analyzed. All the three sequences were clustered with the reference sequences from Yunnan, indicating the same geographic origin of the tested and referenced *M. mulatta* (Fig. 2).

Analysis of alternative splicing isoforms of TRIMCyp transcripts

To investigate the status of the *CypA* retrotransposition in *TRIM5* at the transcriptional level in TRIMCyp individuals, another pair of primers (E2/Ec) that was anchored at the termini of TRIMCyp cDNA was used to investigate the TRIMCyp transcripts by PCR and sequencing (Fig. 1b). This primer pair was firstly used to confirm the chromosomal *CypA* insertion in *TRIM5*. Sequence analysis of intron 6 indicated a G-to-T transition in all TRIMCyp individuals of all species examined, which was believed to lead to the splicing of *TRIM5* to *CypA* when transcribed (Brennan et al. 2007; Newman et al. 2008). Furthermore, the TRIMCyp transcriptional variants were examined. A total of 120 clones of TRIMCyp cDNA amplified from five *M. nemestrina* individuals, 80 clones from three *M. mulatta* individuals and 120 clones from five *M. fascicularis* individuals were sequenced. Three major alternative splicing isoforms

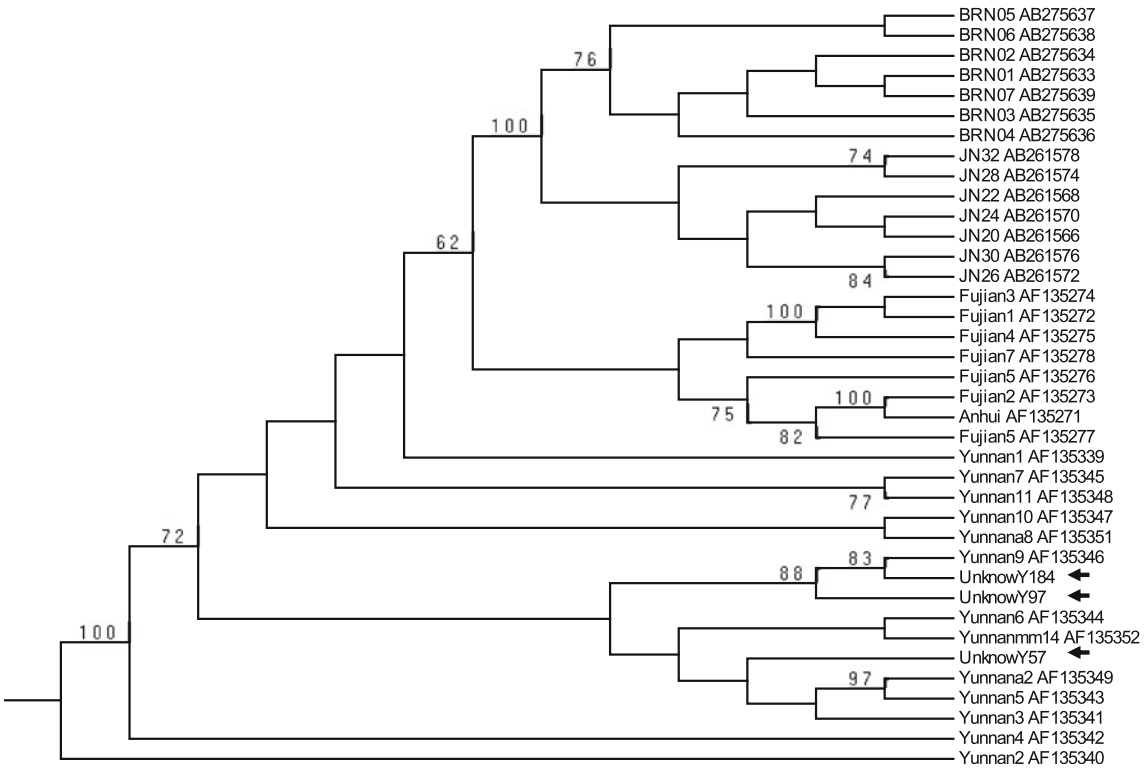

Fig. 2 Phylogenetic analysis of mitochondrial DNA from *M. mulatta* originating from Yunnan with the reference sequences. Mitochondrial DNA sampled from three *M. mulatta* originating from Yunnan Province were cloned and sequenced. These sequences (marked with *arrows*) were phylogenetically analyzed for genetic distance with sequences from Bangladeshi (*BRN*) and Japanese (*JN*) *M. mulatta* and Chinese *M. mulatta* originating from the provinces of, Anhui, Fujian, and Yunnan by using Clustalw and Phylip 3.69 program

were identified: TRIMe4-CypA (E4), TRIMe5-CypA (E5) and TRIMe6-CypA (i.e., TRIMCyp, which is termed E6 only when splicing isoforms are considered in this paper; see Fig. 3). Consistent with other published studies (Brennan et al. 2008; Liao et al. 2007), E6, which is translated into the functional TRIMCyp fusion protein, and E4, which encodes the shortest form of the fusion protein, were the major isoforms, and E6 was expressed at a higher level than E4 in *M. nemestrina* (39.2 vs. 25.8 %) and *M. mulatta* (50 vs. 32.5 %). However, *M. fascicularis* expressed less E6 (45 %) than E4 (50 %). In all three macaque species, E5 was the least expressed isoform of the TRIMCyp transcript. In *M. mulatta*, which had the highest E5 expression of all species studied, E5 accounted for only 7.5 % of TRIMCyp transcripts.

Additionally, the expression of the splicing isoform TRIMe7-CypA (E7) in *M. mulatta* was firstly detected in this study. The E7 isoform was initially reported in *M. nemestrina* in only about 1 % of the total transcripts of TRIMCyp in 2007 (Liao et al. 2007). This isoform is generated due to the ambiguous recognition of the 5′-end of *TRIM5* exon 7 and its splicing with the 3′-end of exon 6, after which the first two nucleotides of exon 7 are deleted (Fig. 4a). Our results showed that E7 was identified in *M. nemestrina* and *M. mulatta* at the ratio of 30 and 10 %, respectively and was not detected in *M. fascicularis* (Fig. 2). Because of the frame shift of exon 7 of the *TRIM5* and *CypA* regions in E7, the deduced C-terminal amino acid sequence of the fusion protein was altered to an arginine-rich sequence and was terminated by a premature stop codon (Fig. 3b).

Analysis of TRIM5α and TRIMCyp polymorphisms

Next, the polymorphisms in TRIM5α and TRIMCyp in these four macaque species were analyzed. Multiple studies have reported that TRIM5α shows intra- and interspecies polymorphism among Old World monkeys (Newman et al. 2006; Sawyer et al. 2005). The polymorphisms of TRIM5α in ten *M. mulatta*, ten *M. thibetana* originating from China, and five *M. fascicularis* from Indochina were investigated in this study, too. Our TRIM5α sequence data were consistent with these findings. As shown in Fig. 5, TRIM5α in *M. mulatta* and *M. thibetana* had abundant polymorphisms that mainly clustered within the coiled-coil domain and SPRY domain. The coiled-coil domain is important for self-trimerization (Javanbakht et al. 2006; Mische et al. 2005), which also affects TRIM5α function (Diaz-Griffero et al. 2008; Perez-Caballero et al. 2005). The SPRY domain is mainly responsible for recognizing the capsid region of invading retroviruses and is thus under strong positive selection driven by retroviruses during evolution (Newman et al. 2006). Polymorphisms within these domains suggest variations in this restriction factor in antiviral activity. No significant intraspecies polymorphisms of *M. fascicularis* TRIM5α were identified in this study. Additionally, it was reported that a 334P to Q change in *M. mulatta* TRIM5α and a 332P to R change in human TRIM5α led to the loss of restriction of HIV-1 replication (Li et al. 2006). In this study, residue 334 was a proline in all of the detected *M. mulatta* samples, which is considered a regional characteristic.

Furthermore, the sequence "QAPGTLFTFPSLT" was detected beginning at position 330 in the *M. mulatta* TRIM5α (Sawyer et al. 2005). This patch of residues was "QSPGTLF—QSLT" in all *M. mulatta* and *M. thibetana* examined in this study due to the deletion of the TF at 339–340. Other groups also reported that both the 339-340TF-deleted and undeleted genotypes were present in *M. mulatta* (Newman et al. 2006). The 339–341TFP in the V1 region of TRIM5α played a vital role on restriction of retrovirus replication (Kono et al. 2008). Thus, the 339–340TF deletion appears to be a regional species-specific phenomenon. Moreover, other polymorphisms, such as 178Y/H, 184S/L, 196W/R, and 222E/K, were also

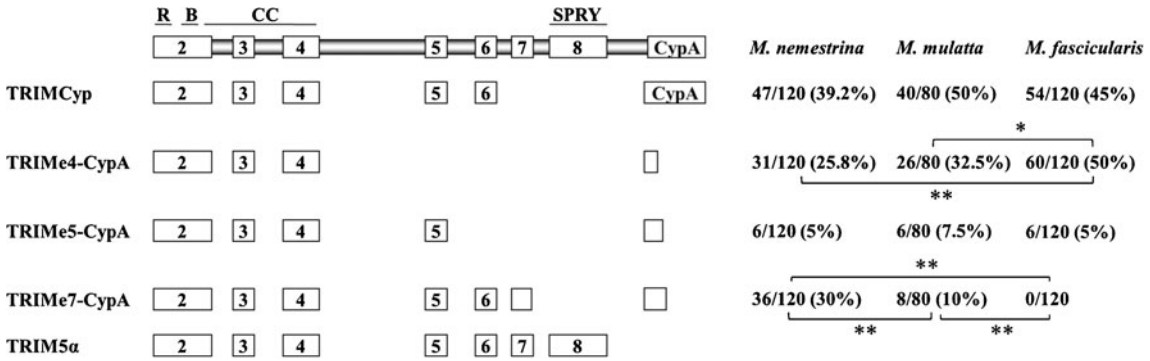

Fig. 3 Analysis of TRIMCyp alternative splicing isoforms. The CypA sequence pseudogene was fused to the TRIM5α species at coding regions for exons 4–7, forming a series of splicing isoforms, which were termed TRIMe4-CypA, TRIMe5-CypA, TRIMCyp, and TRIMe7-CypA. The expression ratios of the splicing isoforms in three species of *M. nemestrina*, *M. mulatta*, and *M. fascicularis* are presented. *$P<0.05$; **$P<0.01$

Fig. 4 The mechanism of TRIMe7-CypA splicing. **a** The 3′-end of intron 6 of *TRIM5* with the retrotransposed *CypA* has an AG-to-AT mutation, while the initial dinucleotide at the 5′-end of exon 7 is AG, which can be misread as the splicing site. **b** Consequently, in TRIMe7-CypA, exon 6 fuses to exon 7, which has a deletion of the first AG at the 5′-end and is followed by the CypA fragment. The reading frame of exon 7 and CypA in E7 is shifted due to the deletion of the AG dinucleotide. The deduced amino acid sequence produced after this splicing is arginine (*R*) rich

identified in *M. mulatta* and *M. thibetana* (Fig. 5). Taken together, our data regarding the polymorphisms in TRIM5α highlighted the inter- and intraspecies complexity of this restriction factor, especially within *M. mulatta* and *M. thibetana*.

In contrast to TRIM5α, TRIMCyp showed more intraspecies polymorphisms in *M. fascicularis* than in *M. mulatta* and *M. nemestrina* (Table 2). Sequencing data showed that the polymorphic regions of *M. fascicularis* TRIMCyp were mainly clustered in the Linker-2 region and the CypA domain. It was reported that polymorphisms in the Linker-2 region of TRIM5α could affect the function of this restriction factor (Sastri et al. 2010). However, the previously reported polymorphisms of Linker-2 were not identified in any of the *M. fascicularis* examined in this study. Instead, two other polymorphic sites in the Linker-2 region, 247E/D and 285R/G, were detected in *M. fascicularis*. Furthermore, three polymorphic sites, 316N/D, 369D/N, and 446K/E, were identified in the CypA domain of TRIMCyp in *M. fascicularis*. It is noticeable the 316N/D polymorphism may be regional specific, because it was not identified in *M. fascicularis* TRIMCyps reported by other studies. In addition, a previous study reported that the polymorphisms of 178Y/H in the coiled-coil region and H357R in the CypA domain could influence the TRIMCyp restriction activity (Dietrich et al. 2011). The residues at these sites were 178H and 357H in all *M. fascicularis* individuals examined in this study.

Recently, TRIMCyp of *M. fascicularis* was further divided into two subtypes, i.e., TRIMCyp (NE) and TRIMCyp (DK), according to polymorphisms at residues 369 and 446. DK was confirmed to be able to restrict HIV-1 replication (Dietrich et al. 2011). The NE haplotype was the minor type and in disequilibrium with a G at the upstream site of 285 (Saito et al. 2012). Therefore, we examined these two subtypes of TRIMCyp in *M. fascicularis* by the direct sequencing of PCR products. As shown in Table 2, of the 160 TRIMCyp forms detected in *M. fascicularis*, seven were TRIMCyp (NE) and 153 were TRIMCyp (DK). This proportion was consistent with a previous report by Saito et al. (2012).

Discussion

In this study, we investigated the distribution of TRIMCyp in four species of macaques from different breeding centers in China. This haplotype was identified in *M. nemestrina*, *M. mulatta*, and *M. fascicularis* but not *M. thibetana*. We identified the TRIMCyp genotype in Chinese *M. mulatta* originating from Yunnan for the first time, although the allele frequency was only 3 %, far lower than the 25 % reported in Indian *M. mulatta* (Wilson et al. 2008). However, we failed to identify the TRIMCyp haplotype in 400 *M. mulatta* originating from Guangxi and Sichuan.

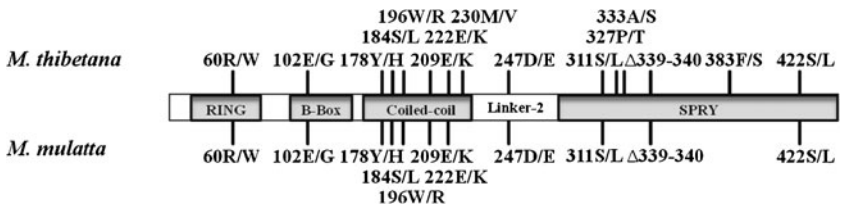

Fig. 5 Intraspecies polymorphism of TRIM5α. The *numbers* indicate the polymorphisms in amino acid sequences of TRIM5α between *M. thibetana* and *M. mulatta*. The coiled-coil domain and SPRY domain are highly polymorphic between these two species

Table 2 TRIMCyp inter- and intraspecies polymorphisms and subgenotypes

Position[a] Species	182	209	247	285	316	369	446	Subgenotype	
Macaca nemestrina	N	E	E	G	D	N	E	5/5	
Macaca fascicularis	N	K	E/D	G/R	N/D	N/D	E/K	7/160	153/160
Macaca mulatta	K	E	D	G	D	N	E	11/11	
Domain	Coiled-coil		Linker-2		Cypa			NE	DK

NE TRIMCyp (NE), *DK* TRIMCyp (DK)

[a]Positions of deduced amino acid sequence

Although all of these rhesus monkeys were from breeding centers, their origin was clearly documented and that of the Yunnan was also confirmed by phylogenetic analysis of the mitochondrial DNA sequences. Furthermore, *M. fascicularis* can be divided into continental (Malaysia and Indochina) and insular (Philippines and Indonesia) population groups based on restriction fragment length polymorphism analysis of mitochondrial DNA (Harihara et al. 1988). The allele frequency of TRIMCyp in *M. fascicularis* originating from Indochina (11.7 %) was considerably lower than the frequency of Malaysian-origin *M. fascicularis* (67.7 %) reported recently (Saito et al. 2012). The reason for this large difference in the prevalence of *CypA* retrotransposition is not clear, and it is an interesting topic for further study.

With the exception of *M. mulatta* from Yunnan examined in this study, there have been no reports describing the TRIMCyp haplotype in Chinese *M. mulatta*. Geographically, Yunnan borders Myanmar, which connects to northern India. It is known that the *Macaque* genus originated from North Africa and migrated to northern India approximately three million years ago and then to Indonesia approximately two million years ago. Studies of the genomic divergence between Indian and Chinese *M. mulatta* subspecies revealed that the genetic differences were as great as those between some primate species. The results of these studies suggest that Indian and Chinese rhesus monkeys were reproductively isolated during the Pleistocene (Smith 2005), most likely because of ancient ecological barriers or the geographic barrier between Bangladesh and Myanmar (Groves 2001). Additionally, Chinese *M. mulatta* can be further divided into four or six subspecies (Fooden 2000; Groves 2001). Notably, based on our results and those reported by others, TRIMCyp was detected in several major species of macaques, including the *M. nemestrina*, Indian *M. mulatta*, *M. fascicularis*, and Assam macaques (*Macaca assamensis*, which originated from the region of Yunnan) (Cao et al. 2011). In contrast, *M. mulatta* and *M. thibetana*, a species of macaque that is morphemically very close to *M. assamensis*, are major macaques in the regions of Guangxi and Sichuan. No TRIMCyp individuals were found in these two species originating from these regions. Therefore, macaques with TRIMCyp are significantly more prevalent in Yunnan, showing a trend of geographical enrichment, which is considered to be a consequence of regional evolution under the selective pressure of certain retroviruses.

Consistent with previously published results (Newman et al. 2006; Sawyer et al. 2005), our data indicated major inter- and intraspecies variation of TRIM5α in macaques, especially in the coiled-coil domain and the N-terminal portion of the SPRY domain in *M. mulatta* and *M. thibetana*. The coiled-coil domain is essential for the polymerization of TRIM5α. Thus, polymorphism in this domain may also reflect adaptation of the arm wrestling between host and pathogens. Recent studies showed that in addition to acting as a restriction factor to retroviruses, TRIM5α is also involved in regulating signal transduction related to innate immunity as a pattern recognition receptor (Pertel et al. 2011). Macaques with the TRIMCyp haplotype, especially homozygotes, do not express normal TRIM5α. Therefore, it will be interesting to investigate whether TRIMCyp is also involved in the signal cascades of the innate immune response. The SPRY domain of TRIM family proteins recognizes and binds to invading viruses. Variations in these domains may represent the consequence of interactions between viral infection and host response. In TRIMCyp, the SPRY domain is substituted by CypA, which also replaces SPRY function. Therefore, variation in the CypA domain is considered to affect the antiviral efficacy of TRIMCyp. Our results showed that the intra-species variation of TRIMCyp was higher in *M. fascicularis* than *M. mulatta* and *M. nemestrina*, especially in the Linker-2 and CypA domains. The biological effects of specific polymorphisms in the TRIMCyp macaque populations should be studied further.

Recent findings showed that TRIMCyp of *M. fascicularis* can be further divided into TRIMCyp (DK) and TRIMCyp (NE) haplotypes depending on the DK/NE status at residues 66 and 143 of the CypA domain. DK-type *M. fascicularis*, but not NE-type *M. fascicularis*, restrict the replication of HIV-1 (Dietrich et al. 2011). This DK/NE polymorphism was detected in *M. fascicularis* examined in this study at a similar ratio. However, we did not find the R-DE form of CypA reported by Yilen et al. (2010), which is consistent with the reports of Hu Lab and Akatsuki Lab (Dietrich et al. 2011; Saito et al. 2012). The presence of the HIV-1-resistant DK haplotype of TRIMCyp in *M. fascicularis* and *A. trivirgatus* implies that primates can increase their resistance to

retroviral infections via enhancing polymorphisms of CypA. The existence of TRIMCyp in some or even all individuals of several species of monkeys strongly demonstrates its role in anti-retrovirus immunity during the evolution of these primates. In most species of monkeys, TRIMCyp does not strictly restrict HIV-1, but it is still able to inhibit the replication of some other retroviruses, such as HIV-2, feline immunodeficiency virus (FIV) and SIVagmTAN (Virgen et al. 2008). In addition, the TRIMCyp (DK) of *M. fascicularis* also restricts HIV-1 replication. This phenomenon implies that the prevalence of certain retroviruses that were closely related to HIV-1, HIV-2, FIV, and SIV positively selected individuals expressing TRIMCyp and that this genotype was inherited thereafter. The genomes of TRIMCyp individuals may retain the integrated proviral genomes or genetic fossils of retroviruses that exerted the selective pressure. Scanning host genomes should reveal traces of such viral infections.

Several types of splicing isoforms of TRIM5α and TRIMCyp have been reported, some of which are unable to restrict viruses. We examined the expression ratios of the TRIMCyp isoforms in *M. nemestrina*, *M. mulatta*, and *M. fascicularis* by analyzing the clones of each isoform (E4, E5, and E6) and found that the ratios of these isoforms were considerably different among these species of macaques. We also identified the E7 isoform in *M. nemestrina* and *M. mulatta* but not *M. fascicularis*. This alternative splicing variant is rarely reported. An arginine-rich domain at the C terminal is generated due to a frameshift, creating a positively charged domain that is presumed to bind to the retrovirus capsid through electrostatic attraction. It has been reported that some proteins of the TRIM family, such as TRIM19 (also known as promyelocytic leukaemia protein), lack the SPRY motif but retain their antiviral function. Conversely, some of the splicing isoforms of TRIM5α do not display the inhibitory activity of the prototypical TRIM5α (Battivelli et al. 2011). Therefore, the actual functions of TRIMCyp isoforms must be investigated further. The biological significance of E7 in *M. nemestrina* and *M. mulatta* is another interesting question.

Macaques expressing TRIMCyp provide a potential novel NHP model for AIDS studies because they lack a crucial barrier for HIV-1 replication in host cells. Another essential barrier for HIV-1 infection in macaques is APOBEC3G. Studies have suggested that HSIV containing SIV *vif* is able to infect and replicate in TRIMCyp homozygous macaques, such as *M. nemestrina* (Hatziioannou et al. 2009; Thippeshappa et al. 2011). Recently, human SAMHD1 was found to be an important restriction protein for HIV-1 replication in macrophages and dendritic cells. SAMHD1 in Old World *M. mulatta* and New World *A. trivirgatus* also inhibits HIV-1 infection (Laguette et al. 2012). Currently, HSIV infection of *M. nemestrina*, all of which are TRIMCyp homozygotes, can result in infection without the clinical symptoms of AIDS. The construction of an HSIV that can overcome SAMHD1 may be needed to improve the viral infection model. Because both the *M. fascicularis* and Chinese *M. mulatta* populations include TRIMCyp homozygous individuals, which exhibit polymorphisms in major motifs and express different patterns of TRIMCyp splicing isoforms, these macaques provide novel options for the development of HIV-1-susceptible NHP models.

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