All Three Variable Regions of the TRIM5 α B30.2 Domain Can Contribute to the Specificity of Retrovirus Restriction

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Recent studies have revealed the contribution of TRIM5 α to retrovirus restriction in cells from a variety of primate species. TRIM5 α consists of a tripartite motif (the RBCC domain) followed by a B30.2 domain. The B30.2 domain is thought to be involved in determination of restriction specificity and contains three variable regions. To investigate the relationship between the phylogeny of primate TRIM5 α and retrovirus restriction specificity, a series of chimeric TRIM5 α consisting of the human RBCC domain followed by the B30.2 domain from various primates was constructed. These constructs showed restriction profiles largely consistent with the origin of the B30.2 domain. Restriction specificity was further investigated with a variety of TRIM5 α s containing mixed or mutated B30.2 domains. This study revealed the importance of all three variable regions for determining restriction specificity. Based on the molecular structures of other PRYSPRY domains solved recently, a model for the molecular structure of the B30.2 domain of TRIM5 α was developed. The model revealed that the variable regions of the B30.2 domain are present as loops located on one side of the B30.2 core structure. It is hypothesized that these three loops form a binding surface for virus and that evolutionary changes in any one of the loops can alter restriction specificity.

Numerous mammalian species have been shown to possess restriction systems that protect against retroviral infection. One example of such an intrinsic restriction factor is Fv1. Mice that carry the Fv1ⁿ allele are resistant to B-tropic murine leukemia virus (B-MLV) infection, whereas mice that have a Fv1^b allele are resistant to N-tropic MLV (N-MLV) infection (20, 30, 51). The Fv1 gene product interacts with the capsid (CA) protein of incoming MLV, where a single amino acid at position 110 plays a crucial role in the interaction (29). Restriction can be overcome by viral challenge at a high multiplicity of infection, a phenomenon called abrogation (3, 11, 15, 58). We have previously shown that Fv1 shares about 60% amino acid sequence identity with the *gag* gene of a human endogenous retrovirus (HERV-L) (7), suggesting that Fv1 could be derived from a member of the mouse endogenous retrovirus MERV-L family (4, 7). It seems likely that Fv1 was selected by its capacity to protect mice from exogenous viral infection.

In primate cells, several cellular factors participate in the life cycle of retroviruses. The class of factors known to block retroviruses in primate cells includes Lv1 and Ref1. The Lv1 and Ref1 factors can restrict lentiviruses in nonhuman primate cells and N-MLV in human cells, respectively (5, 6, 13, 21, 34, 57). Extensive efforts to characterize Lv1 and Ref1 resulted in the identification of TRIM5 α as the principal contributor to both Lv1 and Ref1 activity (22, 26, 53, 63). Rhesus macaque TRIM5 α (rhTRIM5 α) was shown to restrict human immunodeficiency virus type 1 (HIV-1), whereas its human orthologue (huTRIM5 α) did not (53). Both orthologues were, however,

capable of restricting N-tropic MLV in a manner similar to that of Fv1, with TRIM5 α targeting the CA protein of incoming retroviruses, and restriction can be overcome by saturation. In marked contrast to Fv1, which restricts retrovirus after the completion of reverse transcription of the viral RNA (25), TRIM5 α blocks the retrovirus life cycle before or during the reverse transcription step (53).

TRIM5 belongs to the tripartite motif (TRIM) protein family. Members of this family characteristically possess the RING, B-box, and coiled-coil motifs that together are known as the RBCC domain (39). The α -isoform of TRIM5, which is one of five splicing variants of TRIM5, includes another large domain, called the B30.2 domain, following the RBCC domain (40). Another isoform of rhTRIM5, rhTRIM5 γ , which does not contain the B30.2 domain, does not restrict HIV-1 (53), suggesting that the B30.2 domain is important for retrovirus restriction. We have previously shown that the B30.2 domain is exchangeable between human and rhesus macaque TRIM5 α (64). A TRIM5 α chimera, which has the RBCC domain of huTRIM5 α and the B30.2 domain of the rhTRIM5 α , restricted HIV-1, whereas the reciprocal chimera, which has the RBCC domain of the rhTRIM5 α and the B30.2 domain of the huTRIM5 α , did not restrict HIV-1 (55, 64). Furthermore, we and others have shown that the owl monkey TRIM5 locus contains a retrotransposed copy of the cyclophilin A (CypA) gene, resulting in expression of a fusion protein of the RBCC domain with CypA (TRIMCyp) (37, 44). This fusion protein restricts HIV-1, but not a mutant HIV-1 that fails to bind CypA, suggesting that CypA has replaced the function of the B30.2 domain in binding CA (37). Taken together, these data suggest that the B30.2 domain is the main determinant of specificity for retrovirus restriction.

In this study, we amplified exon8 of the TRIM5 α gene, which covers almost the entire B30.2 domain, from 12 different

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primate species, and fused it with the RBCC domain of the human TRIM5 α . The resultant chimeric TRIM5 α s were tested for their restriction activity against HIV-1, simian immunodeficiency virus SIVmac, N-MLV, and B-MLV to examine the specificity of retrovirus restriction by primate TRIM5 α . On the basis of the differences in the restriction patterns observed, we characterized the sites of the B30.2 domain that play important roles in specificity determination. Here, we show that all three previously characterized variable regions (V1, V2, and V3) of the B30.2 domain of primate TRIM5 α (43, 49) play important roles in retrovirus restriction, implying that the B30.2 domain of primate TRIM5 α may have evolved to allow the control of infection by various retroviruses. Modeling studies revealed that these variable regions correspond to loops located on the same surface of the molecule, presumably forming the binding surface to retrovirus capsid.

MATERIALS AND METHODS

Amplification of TRIM5 exon8 by PCR. Whole-blood samples were obtained from 12 primate species: three samples each from chimpanzees (*Pan troglodytes*), gorillas (*Gorilla gorilla*), orangutans (*Pongo pygmaeus*), pigtailed macaques (*Macaca nemestrina*), and one sample each from a tamarin monkey (*Cercopithecus tantalus*), sooty mangabey (*Cercocebus atys*), cotton-top tamarin (*Saguinus oedipus*), emperor tamarin (*Saguinus imperator*), Goeldi's marmoset (*Callimico goeldii*), silvery marmoset (*Callithrix argentata*), squirrel monkey (*Saimiri* sp.), and brown capuchin (*Cebus albifrons*). Samples had been collected in the mid-1980s and cryopreserved since then. Chromosomal DNAs were extracted with a commercial kit (QIAGEN). These DNAs were used as templates in the subsequent PCR. To amplify exon8 of TRIM5 α , we used the following primers: intronF (5'-GTAAGGAGAAAGTCACATTATCA-3') as a forward primer and Trim5rev (5'-TCAAGAGCTTGGTGACACAG-3') as a reverse primer. After purification, the amplified PCR products were subcloned into a pBlunt TOPO vector according to the manufacturer's instructions (Invitrogen), followed by sequencing three independent clones. Isolation of TRIM5 α cDNAs from the human (*Homo sapiens*) and rhesus macaque (*Macaca mulatta*) cell lines, TE671 and LLCMK2, were described previously (63).

Preparation of TRIM5 α constructs and their derivatives. The RBCC domain of human TRIM5 α was amplified by PCR using PfuUltra (Stratagene) with primers TopoTRIM5F (5'-CACCATTGGCTTCTGGAACTCTGG-3') and Exon7-RRYW (5'-CCAGTAGCGTCGGACATCTGTCAGCTCTCTAAA-3'), where RRYW is a linker sequence corresponding to the first four amino acids of the human B30.2 domain. Primate B30.2 domains were amplified from the pBlunt TOPO vectors carrying exon8 sequences of primate TRIM5 α . Oligonucleotide RRYW-exon8ape (5'-CGACGCTACTGGGTTGATGTGACAGTGGCTCCAAA-3'), RRYW-exon8owm (5'-CGACGCTACTGGGTTGATGTGACACTGGCTCCAAA-3'), or RRYW-exon8nwm (5'-CGACGCTACTGGGCTCATGTGACACTGGTTCAGT-3') was used as a forward primer to amplify ape, Old World monkey (OWM), and New World monkey (NWM) B30.2 domains, respectively, and Trim5rev was used as a reverse primer. Equal amounts of the PCR products of the human RBCC domain and primate exon8 gene were mixed, and chimeric TRIM5 α was amplified using PfuUltra with the primer pair TopoTRIM5F/Trim5rev. After purification, PCR products were cloned into the pENTR TOPO vector (Invitrogen) to construct an "entry vector." TRIM5 α constructs in the entry vector were transferred to the delivery retroviral vector (pLGatewayIY) which carries enhanced yellow fluorescent protein (EYFP) with the LR Clonase enzyme mix (Invitrogen) (63).

All amino acid substitution, insertion, or deletion mutants used in this study were synthesized using the QuikChange site-directed mutagenesis kit (Stratagene). The desired point mutation or insertion was situated in the middle of the primer, with 16 to 20 bases of correct sequence on either side. For the internal deletion mutants, primers consisting of 18 to 20 bases on either side of the region to be deleted were synthesized as one oligomer. All TRIM5 α constructs with chimeric B30.2 domains were synthesized by PCR using PfuUltra. Reverse primers to amplify the 5' fragment and a forward primer to amplify the 3' fragment consisted of 12 bases of the end sequence of the 5' fragment and another 12 bases of the start sequence of the 3' fragment. RRYW-exon8 was used as a forward primer to amplify the 5' fragment, and Trim5rev was used as a reverse primer to amplify the 3' fragment. After purification, equal amounts of PCR

products of the 5' and 3' fragments were mixed, and a chimeric B30.2 domain was amplified with the primer pair RRYW-exon8/Trim5rev. The subsequent steps were the same as those for preparing TRIM5 α constructs described above. The sequences of the mutations introduced were verified by DNA sequencing. Oligonucleotide sequences of the primers used to prepare mutants and chimeras are available upon request.

Cells and viruses. *Mus dunni* (mouse) cells and 293T and HT1080 (human) cells were cultivated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. Viruses were generated by transient transfection of 293T cells with three plasmids providing delivery vector, *gag-pol*, and *env* by a conventional CaPO₄ method as described previously (10). Briefly, 1 day before transfection, 293T cells were seeded at 1.5×10^6 cells in 5 ml culture medium on 6-cm-diameter dishes. For preparing the delivery vector viruses, 7 μ g each of pVSV-G (10), pHIT60 (47), and delivery vector plasmids were transfected simultaneously. For preparing "tester" viruses, which carry the CA target for restriction and enhanced green fluorescent protein (EGFP), 7 μ g each of pCSGW (2) and p8.91 (67), pAd-SIV3 (36) and pViG Δ BH SIV/HIV GFP (45), pLNCG (63) and pCIG3 N (10), or pLNCG and pCIG3 B (10) were transfected together with pVSVG to produce vesicular stomatitis virus G-pseudotyped HIV-1, SIVmac, N-MLV, or B-MLV, respectively. At 18 h after transfection, cells were treated with 10 mM sodium butyrate for 6 h to stimulate cytomegalovirus promoter-driven expression. At 48 h after transfection, the virus-containing culture supernatant was harvested, filtered through a 0.45 μ m-pore-size filter, and stored immediately at -80°C .

Restriction assay. Assays for restriction by TRIM5 constructs were carried out as described previously (10). Briefly, 5×10^4 HT1080 or *Mus dunni* cells per well were seeded on a 12-well plate. Sixteen hours later, cells were challenged with delivery vector virus to transduce the TRIM5 α construct and EYFP. Approximately 48 h later, cells were split 1:6, and after a further 16 h, they were challenged with tester viruses to transduce the CA target for TRIM5 α and EGFP. Cells were inoculated with an aliquot of delivery and tester viruses that yielded about 25 to 40% yellow and green cells, respectively, in control cells. Forty-eight hours after the second transduction, cells were harvested, fixed in phosphate-buffered saline (PBS)-3.5% formalin, and examined for EGFP and EYFP expression by fluorescence-activated cell sorting analysis with a fluorescence-activated cell sorter LSR apparatus (Becton Dickinson). Restriction was assessed by comparing the percentage of GFP-positive cells in the YFP-positive or -negative cell populations.

Construction of phylogenetic tree. Amino acid sequences of the exon8 gene of TRIM5 α were predicted from their nucleotide sequences and aligned using CLUSTAL W software with minor manual modifications. Phylogenetic trees were constructed by the maximum-likelihood method in the PHYLIP package on the basis of amino acid sequence alignment.

Western blot. *Mus dunni* cells transduced with or without primate TRIM5 α construct were seeded in 12-well plates and grown to confluence. The cells were washed with PBS twice and lysed with lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris-HCl [pH 8.0]). The protein concentration of each lysate was determined by a Bradford assay (Bio-Rad). Protein (18.2 μ g) was loaded for each sample. The proteins were boiled in sodium dodecyl sulfate-containing loading buffer with 5 mM Tris(hydroxypropyl)phosphine as a reductant and separated by polyacrylamide gel electrophoresis followed by transferring proteins onto a polyvinylidene difluoride membrane. After the incubation of the transferred membrane in blocking buffer (PBS containing 5% milk and 0.1% Tween 20) at 4°C overnight, the membrane was incubated in blocking buffer containing polyclonal anti-TRIM5 α (ab4389; Abcam Ltd.) (1 in 1,000 dilution) or anti- α -tubulin (1 in 10,000 dilution) antibody at room temperature for 1 hour. After the membrane was washed, it was incubated at room temperature for 1 hour in blocking buffer containing horseradish peroxidase-conjugated anti-goat immunoglobulin G antibody (1 in 1,000 dilution) for TRIM5 α detection or horseradish peroxidase-conjugated protein A (1 in 1,000 dilution) for α -tubulin detection. After the membrane was washed, the protein bands were detected using the enhanced chemiluminescence (ECL) system (Amersham).

Modeling. The PRYSPRY domain of protein structure 2FNJ (61) was used as the template for building the model. In order to avoid an unreliable sequence-sequence alignment, the sequence of 2FNJ was run through PsiBLAST (1) with a maximum of seven iterations and inclusion threshold of 0.01. Of the resulting sequences, the most diverse nine (with an E value of 0.001 or better) were aligned using the multiple-sequence alignment program Multal (56) with the original query sequence to form a profile. Exactly the same technique was applied to the sequence of the TRIM5 α B30.2 domain. The same program was run in profile-profile mode to align these two subfamilies. The TRIM5 α and 2FNJ sequences were then extracted to obtain the sequence-sequence alignment

FIG. 1. Amino acid sequences of primate TRIM5 α exon8. Amino acid sequences were predicted from their nucleotide sequences and aligned using Clustal W. The amino acid positions are shown above the human TRIM5 α sequence. All TRIM5 α constructs studied herein have the RBCC domain of the human TRIM5 α , so that the start position of all exon8 sequences is 299. The primate species of origin are shown to the left of the sequences, which are grouped as apes, OWM, and NWM. A dot indicates that the amino acid is identical to that of the human exon8 sequence, and a dash indicates an amino acid deletion. Amino acids substituted or deleted in this study were indicated by boxes, and their positions were shown above each amino acid. The boundaries of three variable regions were indicated on the basis of the previous study of Song and collaborators (49). The double line between positions 396 and 397 indicates the site at which the N- and C-terminal halves of the B30.2 domains were exchanged.

required for building a comparative model. A second group of models was built in the same way using 2FBE (18), and the two groups were superimposed.

Models were built using programs Modeller 8v2 (<http://salilab.org/modeller>) and SwissModel v3.5 (<http://swissmodel.expasy.org>). Models have been depicted here using PyMol v0.98.

Nucleotide sequence accession numbers. The GenBank accession numbers of exon8 gene sequences determined in this study are DQ437595 to DQ437606.

RESULTS

Amino acid sequence alignment of primate B30.2 domains.

To assemble a collection of functional TRIM5 proteins, we amplified exon8 of the TRIM5 α gene, which covers most of the B30.2 domain, from whole-blood samples from a variety of primate species and fused it to the RBCC domain of human TRIM5. The deduced amino acid sequences are compared in

Fig. 1. No variation was seen within species for which three independent samples were available (chimpanzees, gorillas, orangutans, and pigtailed macaques). As reported elsewhere (43, 49), there are three highly variable regions in the B30.2 domain. A short amino acid insertion and a 20-amino-acid duplication were found in the V1 region of rhesus macaque and tantalus monkey B30.2 domains, respectively. The V1 region of sooty mangabey TRIM5 α contains a short insertion similar to that present in the rhesus macaque. The pig-tailed macaque B30.2 domain has glutamine at amino acid position 337 instead of the short insertion found in the rhesus macaque and sooty mangabey proteins, which results in the pigtailed macaque B30.2 domain having the same amino acid length as that of ape B30.2 domain. Proteins from NWMs show a nine-

FIG. 2. Phylogenetic tree of TRIM5 α exon8 from various primates and their retrovirus restriction profiles. The tree was constructed on the basis of the amino acid sequence alignment shown in Fig. 1 with the maximum-likelihood method in PHYLIP software. Values to the right of the tree indicate the restriction activities against HIV-1, SIVmac, and N-tropic (N-MLV) and B-tropic (B-MLV) MLV; the restriction activity is the ratio of the percentage of TRIM5 α -positive cells to that of TRIM5 α -negative cells that were infected. A ratio larger than 0.7 indicates the absence of restriction (white box), whereas a ratio less than 0.3 indicates the presence of restriction (black box). A ratio between 0.3 and 0.7 indicates a restriction activity of an intermediate level (gray box). Each value is shown as an average of the ratios obtained from three independent experiments followed by the standard error of the mean. Agm, African green monkey.

amino-acid deletion within the V1 region with a corresponding nine-amino-acid insertion in the V3 region resulting from an incomplete duplication of V3 sequences. Whereas previous studies reported that the B30.2 domains of several NWMs, including howler monkey and spider monkey, have a triplication in the V3 region (48, 49), the NWMs examined in this study did not have such a V3 triplication.

Phylogeny of the B30.2 domain of primate TRIM5 α and their retrovirus restriction profile. To investigate the evolutionary relationships of the B30.2 domains between each primate species, we constructed a phylogenetic tree of the B30.2 domain. The topology of the tree shown in Fig. 2 is essentially similar to those of primate phylogenetic trees on the basis of housekeeping genes (12, 16, 19, 52). The tree showed large clusters of NWMs and Old World primates, with the latter cluster subdivided into ape and OWM clusters. Therefore, the phylogenetic tree shown in Fig. 2 reasonably reflects the phylogeny of primates.

To investigate the relationship between the phylogeny of the B30.2 domain and functional properties, we examined the ability of our collection of TRIM5 α constructs to restrict HIV-1, SIVmac, N-MLV, and B-MLV (Fig. 2). As expected, B-MLV was essentially unrestricted by any TRIM5 α construct with the possible exception of the chimpanzee TRIM5 α , which gave very slight restriction (less than twofold). Among the apes, considerable variation in restriction profile was observed. Human TRIM5 α restricted N-MLV, but not HIV-1 and SIVmac. The restriction profile of chimpanzee TRIM5 α was essentially similar to that of its human counterpart. By contrast, gorilla and orangutan TRIM5 α constructs restricted HIV-1 and SIVmac as well as N-MLV, although the restriction activity against HIV-1

of the gorilla TRIM5 α was only partial. Among OWMs, the rhesus macaque, pig-tailed macaque, and sooty mangabey TRIM5 α restricted HIV-1 and N-MLV, whereas they did not restrict SIVmac. However, the tamarin monkey protein recognized SIVmac as well as HIV-1 and N-MLV. Finally, in marked contrast to the ape and OWM constructs, almost all of the NWM B30.2 domains studied in this study recognized SIVmac, but most did not restrict HIV-1 and N-MLV. Exceptions were TRIM5 α of tamarin species and capuchin. Cotton-top tamarin and emperor tamarin TRIM5 α restricted HIV-1 and N-MLV in addition to SIVmac, while capuchin TRIM5 α restricted N-MLV, but not HIV-1 and SIVmac. Since all of the TRIM5 α constructs analyzed in this study have an identical human RBCC domain, the observed differences in the restriction activity must be attributed to differences within the B30.2 domains. Thus, the B30.2 domain of TRIM5 α would seem to be involved in determining the specificity for retrovirus restriction.

For the most part, the retrovirus restriction profiles of primate TRIM5 α s seen here reflect those of primate cell lines. For example, the result that the rhesus macaque TRIM5 α restricted HIV-1 and N-MLV, but not SIVmac, is consistent with that from the previous studies, where SIVmac replication, but not HIV-1 replication, was seen in rhesus macaque cells (23). However, in a few cases, the restriction profiles were somewhat different from those previously reported (49). For example, the orangutan TRIM5 α published previously gave only partial restriction of HIV-1 (49). This discrepancy might be attributed to the difference in the RBCC domain of TRIM5 α (i.e., whereas the TRIM5 α proteins reported previously carried the intrinsic RBCC domain, those studied here

FIG. 3. Importance of the V1 region of the B30.2 domain of ape and NWM TRIM5αs for retrovirus restriction. Structures of the chimeras (a and c) and the altered amino acids on the B30.2 domain (b and d) made in this study are shown on the left, while their restriction properties are shown on the right. (a) White and black boxes indicate chimpanzee and gorilla sequences, respectively. (b and d) The amino acid positions corresponding to the human and NWM TRIM5α proteins are indicated above the constructs. (c) White boxes indicate tamarin-derived sequences, while black boxes indicate marmoset-derived sequences. Restriction assays were scored as described in the legend to Fig. 2. Hsa, *Homo sapiens*; Ggo, *Gorilla gorilla*; Soe, *Saguinus oedipus*; Cgo, *Callimico goeldii*.

carried the human RBCC domain), or by the difference in the tag (i.e., whereas the TRIM5α proteins studied previously were tagged with a short fragment of the hemagglutinin protein, those studied here were not tagged).

Importance of the V1 region of the ape B30.2 domains for restriction activity. The retrovirus restriction profiles of ape TRIM5α showed that the gorilla and orangutan TRIM5αs, but not the human and chimpanzee TRIM5αs, recognized HIV-1 and SIVmac (Fig. 2). To examine which part of the B30.2 domain controlled this restriction activity, we compared the chimpanzee and gorilla B30.2 domains. As a first approach, N- and C-terminal halves were exchanged between the chimpanzee and gorilla B30.2 domains to make chimeric B30.2 domains between those two ape species. Whereas the CG chimera, which has an N-terminal half of the chimpanzee B30.2 domain, did not restrict HIV-1 or SIVmac, the GC chimera, which has the N-terminal half of the gorilla B30.2 domain, restricted both (Fig. 3a). This result suggested that the N-terminal half of the

B30.2 domain includes determinants for HIV-1 and SIVmac restriction.

To further characterize these determinants, we compared the amino acid sequences of the N-terminal halves of the chimpanzee and gorilla B30.2 domains. Two amino acids were found to be different (Fig. 1). One of them is in the V1 region (at amino acid position 332). We and others have previously reported that a single amino acid substitution from arginine to proline at position 332 conferred HIV-1 restriction activity to human TRIM5α, suggesting the importance of this amino acid for retrovirus restriction (55, 64). The gorilla B30.2 domain has glutamine at this position. To examine whether the glutamine at position 332 can contribute to retrovirus restriction, this change was introduced into the human and chimpanzee proteins by site-directed mutagenesis (generating Hsa332Q and Ptr332Q, respectively). Both Hsa332Q and Ptr332Q acquired the ability to restrict HIV-1 and SIVmac (Fig. 3b and data not shown). Conversely, Ggo332R, which has arginine instead of

glutamine at position 332 in the context of the gorilla B30.2 domain, lost restriction activity against HIV-1 and SIVmac (Fig. 3b). These data confirmed the importance of the amino acid at position 332 of the ape B30.2 domain for lentivirus recognition (64) and suggested that glutamine at this position plays an important role for the restriction activity of the gorilla B30.2 domain.

Importance of the V1 region for HIV-1 and N-MLV restriction activity in NWMs. Retrovirus restriction activities were found to differ considerably between OWM and NWM TRIM5 α s. The former tend to restrict HIV-1 and N-MLV, and the latter tend to restrict SIVmac but not HIV-1 and N-MLV (Fig. 2). Thus, we sought to identify the region(s) in the NWM B30.2 domain involved in determining restriction specificity. To characterize the sites of the NWM B30.2 domain that are involved in the restriction of HIV-1 and N-MLV, we analyzed the cotton-top tamarin and Goeldi's marmoset B30.2 domains. First of all, the N- and C-terminal halves of the tamarin and marmoset B30.2 domains were exchanged (TM and MT [Fig. 3c]). As expected, both chimeras recognized SIVmac as efficiently as the parental tamarin and marmoset TRIM5 α s did. The TM chimera, which has the N-terminal half of the tamarin B30.2 domain, restricted HIV-1 and N-MLV, whereas the reciprocal chimera MT, which has the N-terminal half of the marmoset B30.2 domain, restricted neither HIV-1 nor N-MLV (Fig. 3c). Those findings suggested that the N-terminal half of the tamarin B30.2 domain includes the molecular determinant(s) for restriction activity. Then, we exchanged the V1 and V2 regions between the tamarin and marmoset B30.2 domains in the context of the MT chimera (TMT and MTT [Fig. 3c]) to examine which variable region is important for restriction activity. Whereas the V2 region of the tamarin B30.2 domain did not confer restriction activity to the MT chimera, the TMT chimera, which has the V1 region of the tamarin B30.2 domain, restricted HIV-1 and N-MLV as well as the tamarin TRIM5 α and the MT chimera did. Those results suggested that the N-terminal region of the tamarin B30.2 domain, which includes the V1 region, is important for restricting HIV-1 and N-MLV.

An amino acid comparison showed that in the N-terminal region of the B30.2 domain, four species-specific differences exist between tamarins and marmosets (Fig. 1 and 3c). Among those four amino acids, we focused on the amino acid at position 334 (asparagine on the tamarin B30.2 domain and lysine on the marmoset B30.2 domain), since lysine has a positive charge. We mutated asparagine to lysine at this position on the tamarin B30.2 domain (Soe334K) and lysine to asparagine on the marmoset B30.2 domain (Cgo334N) by site-directed mutagenesis. Soe334K lost restriction activity against HIV-1 and N-MLV, while Cgo334N acquired restriction activity against N-MLV (Fig. 3d). Cgo334N, however, did not acquire restriction activity against HIV-1 (Fig. 3d). Those data suggest that while the amino acid at position 334 is responsible for N-MLV restriction, it is important, but not sufficient, for HIV-1 restriction.

The observation that Cgo334N did not restrict HIV-1 could be due to the C-terminal half and/or the V2 region of the tamarin B30.2 domain being needed for restricting HIV-1 in conjunction with the amino acid at position 334. Alternatively, a change in an additional amino acid(s) in the N-terminal

region might be required for efficient HIV-1 restriction. To test the first possibility, we mutated arginine to lysine at position 334 on the MT and MTT chimeras, which have the V3 and V2-V3 regions of the tamarin B30.2 domain, respectively (Fig. 3c). The resultant mutants, MT 334N and MTT 334N, failed to restrict HIV-1 (data not shown). Therefore, the V2 and V3 regions seemed not to be involved in recognizing HIV-1. To examine the second possibility, two additional amino acids, valine at position 324 and leucine at 330, were substituted with phenylalanine and serine on the marmoset B30.2 domain, respectively (Fig. 3d). The resultant mutant, Cgo324F/330S/334K, acquired restriction activity against HIV-1. Unexpectedly, while a single amino acid substitution from asparagine to lysine at position 334 in the context of the tamarin B30.2 domain resulted in loss of restriction activity by the tamarin TRIM5 α against HIV-1 and N-MLV (Soe334K [Fig. 3d]), another single amino acid substitution from phenylalanine to valine at position 324 in the context of the tamarin B30.2 domain resulted in the tamarin TRIM5 α which lost the ability to restrict HIV-1 but still retained restriction activity against N-MLV (Soe324F) (Fig. 3d). Those results suggest that a limited set of amino acids in the V1 region of the NWM B30.2 domain play a crucial role on HIV-1 and N-MLV restriction. Taken together, the importance of the V1 region of the B30.2 domain is evident for both ape and NWM TRIM5 α s.

Importance of the V2 region of the orangutan B30.2 domains for HIV-1 and SIVmac restriction. Since the orangutan B30.2 domain, like the gorilla B30.2 domain, has glutamine at position 332 and restricted HIV-1, SIVmac, and N-MLV, glutamine at this position was replaced with arginine (Ppy332R [Fig. 4]). Unexpectedly, Ppy332R retained full restriction activity against HIV-1 and partial activity to SIVmac, suggesting the involvement of other amino acids in the V1 region or other variable regions of the orangutan B30.2 protein for retrovirus restriction. Sequence comparison between the gorilla and orangutan N-terminal halves of the B30.2 domain revealed differences at 11 amino acids. Among those 11 amino acids, we focused on the amino acids at four positions that have different charges (positions 307, 324, 335, and 389). These include aspartic acid, glutamic acid, threonine, and glutamine from the orangutan B30.2 domain, which correspond to asparagine, lysine, arginine, and lysine, respectively, in the gorilla B30.2 domain (Fig. 1). We mutated three amino acids in the N-terminal region (at positions 307, 324, and 335) and one amino acid in the V2 region (at position 389) on the orangutan B30.2 domain independently (Ppy-V1 and Ppy389K, respectively). However, both Ppy-V1 and Ppy389K retained restriction activity against HIV-1 and SIVmac (Fig. 4). Next, we combined the glutamine-to-arginine substitution at position 332 and the glutamine-to-lysine substitution at 389 (Ppy332R/389K) (Fig. 4). This orangutan TRIM5 α variant with the double mutation lost restriction activity against HIV-1 and SIVmac (Fig. 4). The V1 region mutations were combined with the glutamine-to-lysine substitution at 389, generating Ppy-V1/389K. Whereas this orangutan variant retained SIVmac restriction activity, it lost the ability to restrict HIV-1 (Fig. 4). When the V1 region mutations were combined with the glutamine-to-arginine substitution at position 332 (Ppy-V1/332R), restriction activity against HIV-1 and SIVmac of Ppy-V1/332R was weakened but was not fully lost (Fig. 4). We substituted glutamine at position

FIG. 4. Importance of the V2 region of the B30.2 domain of orangutan TRIM5α for HIV-1 and SIVmac restriction. Structures of the B30.2 domain mutants are shown on the left, while the restriction properties of the variants on HIV-1, SIVmac, and N-MLV are shown on the right. The amino acid positions corresponding to the orangutan TRIM5α protein are indicated above the constructs. Restriction assays were scored as described in the legend to Fig. 2. Ppy, *Pongo pygmaeus*.

389 with lysine in the context of Ppy-V1/332R, generating Ppy-V1/332R/389K. This construct lost restriction activity against HIV-1 but retained SIVmac restriction activity (Fig. 4). Although amino acids of Ppy-V1/389K and Ppy-V1/332R/389K at positions 307, 324, 335, and 389 are the same as those of the gorilla and Ggo332R at the same positions, respectively, the restriction activities against HIV-1 and SIVmac were different for Ppy-V1/389K and gorilla and for Ppy-V1/332R/389K and Ggo332R (Fig. 3 and 4). These observations suggest that other amino acids in the B30.2 domain could be involved in determining restriction specificity. We focused on an amino acid at position 385 in the V2 region (cysteine in the higher ape B30.2 domain and tyrosine in the orangutan B30.2 domain), since cysteine and tyrosine at this position are well conserved among the B30.2 domains of higher apes and OWMs, respectively (Fig. 1). Substitution of tyrosine with cysteine at position 385 in the context of Ppy389K (Ppy-V2) did not change the restriction activity of Ppy389K, suggesting that amino acid substitution in the V2 region alone cannot alter the restriction activity of the orangutan TRIM5α construct. The tyrosine-to-cysteine substitution at position 385 diminished the SIVmac restriction activity by Ppy-V1/332R/389K (Ppy-V1V2/332R [Fig. 4]). Therefore, the amino acid residue at position 385 is also involved in determining the restriction specificity of the orangutan B30.2 domain. On the basis of these results, a role for the V2 region for HIV-1 and SIVmac restriction was revealed when a mutation in the V2 region was combined with changes in the V1 region.

Importance of the V3 region of the NWM B30.2 domain for HIV-1 and SIVmac restriction. The retrovirus restriction profiles of NWM TRIM5αs suggested that NWM B30.2 domains tend to interact with SIVmac (Fig. 2). To identify the regions in the NWM B30.2 domain important for SIVmac restriction, we exchanged the N- and C-terminal halves of the B30.2 domains between chimpanzee and Goeldi's marmoset TRIM5αs (CM and MC [Fig. 5a]), since the chimpanzee TRIM5α restricted N-MLV, but not HIV-1 and SIVmac. In marked contrast to the previous studies with the chimpanzee-gorilla chimera and the tamarin-marmoset chimera (Fig. 3), the SIVmac

restriction specificity of this chimera mapped to the C-terminal region (Fig. 5a). Therefore, the C-terminal half of the marmoset B30.2 domain contains the molecular determinants for SIVmac restriction. In addition, the chimera CM restricted N-MLV as strongly as the chimpanzee TRIM5α construct, whereas the restriction activity to N-MLV of the reciprocal chimera MC, which contains the N-terminal half of the marmoset B30.2 domain, was as weak as the restriction activity to N-MLV of the marmoset TRIM5α (Fig. 5a). Therefore, restriction activity to N-MLV tends to associate with the origin of the N-terminal half of the B30.2 domain, implying the importance of the N-terminal half of the B30.2 domain for N-MLV restriction. This idea is consistent with the results obtained from the analysis of the chimeras between tamarin and marmoset B30.2 domains described above (Fig. 3).

Since the N- and C-terminal halves of the chimpanzee and marmoset B30.2 domains were exchanged between the V2 and V3 regions, the C-terminal half of the B30.2 domain of the CM chimera contained the V3 region of the marmoset B30.2 domain (Fig. 1). The V3 region of the NWM B30.2 domain has a nine-amino-acid insertion (Fig. 1) compared to those of OWMs. To examine the contribution of this insertion sequence to SIVmac restriction, the nine-amino-acid insertion sequence of the marmoset B30.2 domain was put into the V3 region of the chimpanzee B30.2 domain at the corresponding position. In addition, the three amino acids prior to the nine-amino-acid insertion are different between the chimpanzee and marmoset B30.2 domain sequences (arginine-asparagine-alanine on the marmoset B30.2 domain compared to glutamic acid-glutamic acid-glycine on the chimpanzee B30.2 domain). Therefore, those three amino acids of the chimpanzee B30.2 domain (glutamic acid-glutamic acid-glycine) were substituted with the corresponding three amino acids of the marmoset B30.2 domain (arginine-asparagine-alanine) (Ptr-V3 [Fig. 5b]). As expected, Ptr-V3 still recognized N-MLV as well as the parental chimpanzee TRIM5α. Although it acquired restriction activity against SIVmac, the activity of Ptr-V3 was not as strong as the marmoset TRIM5α. Two amino acids were different in a nine-amino-acid sequence following the nine-amino-acid insertion,

FIG. 5. Importance of the V3 region of NWM B30.2 domain for retrovirus restriction. Schematic representations of the chimeras between chimpanzee and Goeldi's marmoset (a) and of their variants with an insertion and point mutations (b) are presented on the left, while their ability to restrict HIV-1, SIVmac, and N-MLV is shown on the right. (a) Chimpanzee sequences are represented by black boxes, whereas those of marmoset are represented by white boxes. (b) A dot indicates that the amino acid is identical to that in the chimpanzee exon8 sequence, and a dash indicates an amino acid deletion. The amino acid positions corresponding to the chimpanzee TRIM5α protein are indicated above the constructs. Restriction assays were scored as described in the legend to Fig. 2. Ptr, *Pan troglodytes*; Cgo, *Callimico goeldii*.

which constitutes a duplication together with the inserted nine-amino-acid sequence, in the chimpanzee and marmoset sequences (at positions 410 and 412) (Fig. 5b). Those two amino acids in the chimpanzee sequence were substituted with the corresponding ones of the marmoset sequence in Ptr-V3, resulting in Ptr-V3 410Y/412D. This molecule restricted SIVmac as strongly as the marmoset TRIM5α did (Fig. 5b). Conversely, when the nine-amino-acid duplication was deleted from the marmoset B30.2 domain (Cgo-V3), restriction activity against SIVmac was lost (Fig. 5b). Since the MC chimera and the marmoset Cgo-V3 variant did not restrict any of the retroviruses tested, we examined protein expression of these variants using a polyclonal antibody specific to TRIM5 protein. As shown in Fig. 6, a specific band was detected for both MC and Cgo-V3, indicating that the observed lack of activity was not a result of altered expression. We conclude that the duplicated sequence in the V3 region of NWM B30.2 domain plays an important role for retrovirus restriction.

FIG. 6. Protein expression levels of the MC chimera and the Cgo-V3 mutant. Expression levels in extracts of control cells and cells transduced with vectors encoding huTRIM5, chimera MC, and mutant Cgo-V3 were examined by Western blotting using sera specific for TRIM5α (top) and α-tubulin (bottom). The arrow indicates the TRIM5-specific band. Cgo, *Callimico goeldii*.

Molecular structure model for the B30.2 domain of primate TRIM5α. The molecular structures of other SPRY and PRYSPRY domains have been solved recently (18, 33, 61). Those studies revealed that conserved amino acid residues found on β-strands could constitute the core structure of the domains (18, 41, 61). This observation suggests that the molecular structure of the B30.2 domain of primate TRIM5α could be similar to those of other B30.2 and PRYSPRY domains (61). On the basis of the published molecular structures, we developed a model for the molecular structure of the B30.2 domain of primate TRIM5α (Fig. 7). A basic feature of the predicted structure is that the two layers of β-sheets are located face to face, forming a distorted sandwich-like core structure (Fig. 7a). The two β-sheet layers were bridged by loops, which protrude from the core structure of the B30.2 domain. In our model, all variable regions that were found to be important for retrovirus recognition were assigned to the loops that formed a surface of the B30.2 domain core structure (Fig. 7b). This observation seems reasonable, given that a similar surface area of the SPRY domain of GUSTAVUS protein is reportedly important for binding with its ligand (61).

The coordinates of the β-sheets remained constant across all models, regardless of which structural template was used (2FBE and 2FNJ). However, there was considerable variation between models in the positions of the loops, and V1 was particularly varied, adopting conformations which separated the same residue by up to 30 Å. This highlights the differences possible when more than one template is used to build a model, but it also demonstrates the freedom of long loop regions to be arranged in space.

The three loops on the side of the beta-sandwich opposite to the binding surface were also examined. They are 7, 4, and 10 amino acids long and are therefore too short to extend to the binding surface and give the beta-strand arrangement. Among

FIG. 7. Model for the structure of the B30.2 domain of primate TRIM5 α based on the molecular structure of other SPRY and PRYSPRY domains reported previously (61). (a) Ribbon model showing β -sheets. (b) Filled model showing a potential binding surface. Red, green, and blue colors indicate the V1, V2, and V3 regions, respectively. Amino acids that were found to be important for retrovirus restriction are highlighted.

the 14 primate species, the length of each loop is consistent, and although there is a small degree of sequence variability, the amino acid conservation is generally much higher than on the binding interface. This is in contrast to the longer, flexible loops on the binding surface which show a much higher degree of sequence variability in the primate species. Taken together, all of the variable regions of the B30.2 domain of primate TRIM5 α lie on one surface of the molecule, presumably forming a binding surface for interacting with retroviral capsid.

DISCUSSION

It is thought that the B30.2 domain of TRIM5 α protein is involved in recognition and binding of the virus capsid core (54, 62). This concept is supported by studies of other SPRY and PRYSPRY domain-containing proteins showing that this domain is important for interaction with their ligands (24, 61, 66). We have now characterized the sites of the B30.2 domain of primate TRIM5 α important for retrovirus restriction. This study revealed that all variable regions of the domain can play

significant roles in retrovirus restriction. The model developed for the molecular structure of the B30.2 domain suggested that all of the variable regions can be assigned to loops, which bridge two β -sheet layers, and presumably form the ligand-binding surface of the molecule. This implies that these variable regions have evolved to generate a surface for binding various retroviruses. However, we cannot exclude the possible involvement of other TRIM5 α motifs, such as the coiled-coil region in specificity determination as is suggested by our previous study of MLV specificity (64), since the present study included only the B30.2 domain of primate TRIM5 α . This may explain the differences in the restriction profiles of some of our TRIM5 α constructs from those of TRIM5 α s previously examined (Fig. 2) (49).

This study showed that glutamine at position 332 in the V1 region of the ape B30.2 domain is an important determinant of restriction (Fig. 3), consistent with our previous study that showed that a single amino acid change to proline at position 332 conferred the ability to restrict HIV-1 upon human TRIM5 α (64). Whereas macaque, olive baboon, and African green monkey TRIM5 proteins have a proline at position 332, the majority of the OWM and ape B30.2 domains studied so far have glutamine at that position (Fig. 1) (43, 49). This finding suggests that the ancestral amino acid at this position may be glutamine. Following the separation of orangutan lineage, an ancestor of the gorilla may have acquired several amino acid changes in the V1 and V2 regions (Fig. 1). Since those amino acid changes did not include the amino acid change at position 332, the gorilla TRIM5 α may have retained an activity to restrict HIV-1- and SIVmac-like viruses. However, after the split of gorilla lineage, an ancestor of the chimpanzee may have acquired the glutamine-to-arginine mutation at position 332, with resulting loss of the ability to restrict HIV-1 and SIVmac.

The V1 region of the B30.2 domain also plays an important role in virus recognition by OWM and NWM TRIM5 α s. We previously reported that transfer of a short insertion in the V1 region of the rhesus macaque B30.2 domain (amino acid positions 336 to 343) to the human B30.2 domain at the corresponding position conferred HIV-1 restriction activity to the human TRIM5 α (64). In addition, the 20-amino-acid insertion in the V1 region of the African green monkey B30.2 domain is reportedly important for SIVmac restriction (35). The specific combination of amino acids at positions 324, 330, and 334 plays an important role in determining the specificity of restriction by NWMs (Fig. 3d). However, although the V1 region is vital, it is not the only region of importance. In apparent contrast to the OWM and ape B30.2 domains, the V3 region of the NWM B30.2 domain plays a significant role in retrovirus recognition (Fig. 5). The NWM B30.2 domain may have acquired a nine-amino-acid insertion in its V3 region, so that they could recognize viruses with SIVmac-like capsid core. In addition, at least one change in the V2 region can modulate the restriction specificity conferred by the V1 region (Fig. 4). Taken together, these data suggest that restriction specificity is determined by the combination of different sequences in the V1, V2, and V3 regions. The variable regions of primate TRIM5 α may have evolved independently to recognize various retroviruses but sometimes arriving at different solutions to recognize the same virus.

It seems likely that TRIM5 α plays an important role in determining the current patterns of sensitivity to various retroviruses in multiple species (8, 17), but what drove this evolutionary process remains an open question. It seems unlikely that the variable regions of the NWM B30.2 domain have evolved to combat lentivirus, since seroepidemiological surveys have not yet identified any case of SIV infection among NWMs (38). The calculation of synonymous amino acid change/non-synonymous amino acid change ratio showed that primate TRIM5 α has evolved under positive pressure (43, 48). Since it has been reported that some types of endogenous retroviruses are detected in a lineage-specific manner (14, 27, 28, 32, 60, 65), it has been suggested that primate TRIM5 α may have evolved to control specific endogenous retroviruses. However, it seems more likely that exogenous viruses would provide a greater selective pressure than endogenous retroviruses would. Thus, the important driving force for TRIM5 α change may well correspond to no longer extant exogenous retroviruses that have perhaps been preserved as endogenous fossils. Future studies may reveal the relationships between functional evolution of the B30.2 domain and its restriction activity against endogenous retroviruses.

Given the dramatic changes in TRIM5 α sequence seen during evolution (43, 48), it is tempting to conclude that variation in TRIM5 α might account for differential sensitivity to retroviral infection within a species. However, recent studies have not shown any correlation between polymorphisms in human TRIM5 α gene and restriction activity in vitro and serological status in HIV-1-infected patients (42, 50). Therefore, it seems unlikely that TRIM5 α coding sequence variation can explain major differences in HIV-1 infection or progression rates in humans. On the other hand, there seems to be some evidence for such polymorphisms in OWM species. The amino acid sequence alignment data of the B30.2 domain of the TRIM5 α s from macaque species indicate that the amino acid length of the V1 region of the pig-tailed and crab-eating macaque B30.2 domains is the same as that of the V1 region of the ape B30.2 domains, whereas rhesus and Assamese macaque B30.2 domains have a two-amino-acid insertion in their V1 region (Fig. 1) (31, 35). Furthermore, very recent sequencing studies have revealed extensive polymorphism within the coiled-coil and B30.2 domains of TRIM5 α from individual animals from populations of rhesus macaques and sooty mangabeys (W. Johnson, personal communication). It has been reported that the susceptibility of pig-tailed macaques to a chimeric simian/human immunodeficiency virus strain (SHIV_{DH12}) is lower than that of rhesus macaques (46) and that disease progression after inoculation of a pathogenic SIV strain (SIV/ΔB670) in Indian and Chinese rhesus macaques was different (59), and it will be interesting to compare virus susceptibility in members of the same species carrying different alleles of TRIM5 α as a direct test of the in vivo importance of TRIM5 α in determining virus susceptibility.

Most of the mutations that alter the restriction properties of TRIM5 α potentially alter the charge of amino acids in the variable regions of the B30.2 domains. For example, a single amino acid substitution of a negatively charged amino acid (arginine) with a noncharged amino acid (proline or glutamine) at position 332 dramatically changed the restriction activity of the human and chimpanzee TRIM5 α constructs

against HIV-1 and SIVmac (Fig. 3). Such charge changes might play some role in the ability of the B30.2 domain to recognize retroviruses. This is reminiscent of our previous finding that a single mutation from a negatively charged amino acid to a noncharged amino acid at position 358 allowed Fv1ⁿ derivatives to restrict N-MLV and that introduction of a non-charged alanine residue at MLV capsid position 110 renders MLV susceptible to both Fv1ⁿ and Fv1^b (9). However, the importance of charge in the variable regions of the B30.2 domain for retrovirus recognition remains unclear without a precise model for the molecular structure of the domain, since, for example, we are unable to deduce from our model how close to each other the variable loops lie. Our study also showed that the chimpanzee TRIM5 α successfully acquired the ability to restrict SIVmac when a NWM-derived stretch of nine amino acids is inserted in its V3 region, while retaining the restriction activity to N-MLV (Fig. 5). It seems possible that the inserted nine-amino-acid sequence may involve the molecular determinant(s) of the restriction activity against SIVmac. Alternatively, this sequence and point mutations in the following region (C410Y and A412D) may alter the structure of the B30.2 domain. Future structural analyses of TRIM5 α protein would uncover the importance of charges and the impact of the inserted sequences in the variable regions of the B30.2 domain on the structure of the domain, which would provide us a clue to how TRIM5 α recognizes retroviral cores. Finally, the observation that changes in different discrete regions can have the same effect on restriction specificity raises the possibility of trying to identify variable region-binding compounds of potential therapeutic value that retarget human TRIM5 α to recognize HIV-1.

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