Mammalian cells express several factors that act in a cell-autonomous manner to inhibit retrovirus replication. Among these are the Friend virus susceptibility factor 1 (Fv1), lentivirus susceptibility factor 1 (Ref1), and lentivirus susceptibility factor 1 (Lv1). These factors act in a cell-autonomous manner to inhibit retrovirus replication.

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Although primate lentiviruses are normally insensitive to Fv1 and Ref1, HIV-1 and macaque simian immunodeficiency virus (SIVmac) exhibit clearly distinct tropism for primate cells (22, 23), even if entry blocks are overcome by pseudotyping (24). Importantly, resistance to primate lentiviruses is dominant in interspecies heterokaryons, and restriction determinants lie within CA (21, 25–31). These characteristics are reminiscent of those observed for Fv1- and Ref1-mediated N-MLV restriction and imply the presence of restriction factors in primates that block infection by lentiviruses. These factors are collectively referred to as lentivirus susceptibility factor 1 (Ref1) in the nonmurine manner to inhibit retrovirus replication. Among these are the Friend virus susceptibility factor 1 (Fv1), lentivirus susceptibility factor 1 (Ref1), and lentivirus susceptibility factor 1 (Lv1). These factors act in a cell-autonomous manner to inhibit retrovirus replication. Among these are the Friend virus susceptibility factor 1 (Fv1), lentivirus susceptibility factor 1 (Ref1), and lentivirus susceptibility factor 1 (Lv1). These factors act in a cell-autonomous manner to inhibit retrovirus replication.

Recently, a screen of a rhesus monkey cDNA library identified tripartite interaction motif 5α (TRIM5α), a cytoplasmic body component recently shown to block HIV-1 infection in rhesus macaque cells, and can indeed block infection by widely divergent retroviruses. Depletion of TRIM5α from human cells relieved restriction of N-tropic murine leukemia virus (N-MLV), and the expression of human TRIM5α in otherwise nonrestricting cells conferred specific resistance to N-MLV infection, indicating that TRIM5α is Ref1 or an essential component of Ref1. TRIM5α variants from humans, rhesus monkeys, and African green monkeys displayed different but overlapping restriction specificities that were quite accurately predicted by the restriction properties of the cells from which they were derived. All TRIM5α variants could inhibit infection by at least two different retroviruses, and African green monkey TRIM5α was able to inhibit infection by no less than four divergent retroviruses of human, non-human primate, equine, and murine origin. However, each TRIM5α variant was unable to restrict retroviruses isolated from the same species. These data indicate that TRIM5α can confer broad innate immunity to retrovirus infection in primate cells and is likely to be an important natural barrier to cross-species retrovirus transmission.
tional analysis of these TRIM5α variants revealed that TRIM5αhu is responsible for the retrovirus restriction activity previously termed Ref1 and that several TRIM5α isoforms are capable of inhibiting both lentiviruses and N-MLV. Therefore, Ref1 and Lv1 are indeed variants of a single polymorphic inhibitor of retrovirus infection.

Materials and Methods

Cell Lines. Adherent cell lines from humans (HeLa, TE671, and HOS), rhesus monkeys (FRhK4), AGMs (CV-1), mice (Mus dunni) tail fibroblasts, MDTF, and cats (CRFK) were grown in DMEM/10% FCS/antibiotics. The rhesus monkey T cell line, 221, was grown in RPMI medium 1640/20% FCS/antibiotics, supplemented with 10 units/ml IL-2.

Construction of TRIM5α Expression Vectors. Total RNA, extracted from human and monkey cell lines by using TRIzol, was reverse transcribed by using random hexamers and Superscript III reverse transcriptase (Invitrogen). TRIM5α variants were amplified from each of these cDNAs by using PCR primers derived from the 5' and 3' ends of the human TRIM5α coding sequence and were appended with sequences encoding the XhoI and SalI restriction sites. The PCR products were digested with XhoI and SalI and inserted in Bluescript KS+ (Stratagene) or the retroviral expression vector LNCX2 (Clontech). The complete sequence of two to four clones of each PCR product was determined, and representative clones were selected for functional studies. These clones were also inserted into a vector (pCR3.1/HA) that introduces an amino-terminal HA epitope tag. These plasmids were transfected in 293T cells, and cell lysates were analyzed by Western blot to confirm that each TRIM5α protein was expressed at approximately equivalent levels (data not shown).

Viruses and Vectors. HIV-1, SIVMAC, SIVAGMTan, SIVAGM Sab, EIAV, N-MLV, and B-MLV reporter viruses or vectors that carried a GFP-reporter gene were generated by using combinations of either two or three expression vectors. In most cases, Gag-Pol was encoded on a separate expression plasmid from the packaged viral genome; for SIVAGM and SIVAGMSab, reporter viruses were generated by using plasmids that encoded the GFP reporter and Gag-Pol on a single packaged genome (21). HIV-1 and SIVMAC GFP-reporter viruses and vectors generated by two or three plasmid expression systems had identical properties with respect to restriction and were used interchangeably in this study. Details of the reporter virus, Gag-Pol, and vector expression plasmids have been published previously (21, 26). In all cases, viruses and vectors were pseudotyped with vesicular stomatitis virus glycoprotein to enable efficient entry into the mammalian cell lines used in these experiments. Virus/vector stocks were made by transient transfection of 293T cells as described in refs. 21 and 26 and quantitated by infectivity titration on nonrestricting CRFK or MDTF cells and/or by reverse transcriptase assay (Cavidi Tech, Uppsala, Sweden).

Measurement of Retrovirus Restriction Activity. 293T cells were transfected with LNCX2-based retroviral vectors containing either a TRIM5α or an Fv1 cDNA, along with MLV Gag-Pol and vesicular stomatitis virus glycoprotein expression plasmids. Vector stocks produced by these cells were used to transduce MDTF and/or CRFK cells. The transduced cells were selected in 1 mg/ml G418 for 7–10 days and then used as a pool of target cells to test sensitivity to retrovirus infection. These cells were seeded in 24-well plates at 2 × 10⁴ cells per well and inoculated with GFP-reporter virus or vector stocks in the presence of 5 μg/ml polybrene. The virus dose was selected so as to infect 20–50% of unmodified cells. GFP-positive cells were enumerated 48–72 h later by using a FACS Calibur instrument (Becton Dickinson).

RNA Interference. Synthetic short interfering RNA (siRNA) oligonucleotide duplexes were targeted to sequences within human TRIM5 (GGUCAGGGAGGUCAGUUG, [siRNA-A], and GCCUAGCAACUGAAAC, [siRNA-B]) (32). HeLa cells were mock transfected or transfected with 60 pmol of each TRIM5α-specific RNA duplex or a control firefly luciferase duplex (33) by using Lipofectamine 2000 (Invitrogen) and were replated 24 h later. Cells were inoculated 48 hours after transfection with GFP-reporter viruses, and infected cells were enumerated by using a fluorescence-activated cell sorter after another 48 h, as described above. To confirm that the siRNA duplexes could silence TRIM5αhu expression, they were cotransfected with plasmids expressing HA-TRIM5αhu and GFP in HeLa cells. The abundance of these proteins was assessed by Western blotting 48 h later.

Results

Intra- and Interspecies Variation in TRIM5α. We cloned TRIM5α alleles from a number of human, rhesus monkey, and AGM cell lines. An alignment of representative sequences is shown in Fig. 1A. Human TRIM5α variants from TE671 and HOS cell lines were identical to each other and the consensus database sequence and were designated TRIM5αhu,1, but the TRIM5α sequence from HeLa cells differed at a single amino acid position (R136Q) and was designated TRIM5αhu,2. One TRIM5α sequence obtained from rhesus monkey FRhK4 cells differed from the published sequence (32) at a single amino acid position (T307P) and was designated TRIM5αrh,1, whereas a variant present in rhesus 221 cells was the same as the published sequence at this position but differed at two other positions (S184L and W196R). This variant was designated TRIM5αrh,2. Finally, a clone amplified from CV-1 cell cDNA, designated TRIM5αAGM,1, differed from both human and rhesus monkey sequences at multiple positions and contained a 20-aa insertion relative to human TRIM5α variants (Fig. 1A).

Rhesus and AGM TRIM5α Inhibit HIV-1 Infection in Non-Primate Cells. Because human and non-human primate cells express endogenous Ref1 and Lv1 activity that could complicate a functional analysis of TRIM5α mediated restriction, we first asked whether an HIV-1-restricting form of TRIM5α was active in the context of a non-primate cell. Two non-primate cell lines, namely murine MDTF cells, which are Fv1-null, and feline CRFK cells, were used because they are largely devoid of retrovirus restricting activities (18, 20, 34) (data not shown), and all of the retrovirus used in this study exhibit high titer infection therein. As can be seen in Fig. 1B and C, TRIM5αrh,1 conferred resistance to infection by an HIV-1 vector when expressed in murine and feline cells. The level of resistance to HIV-1 conferred by TRIM5αrh,1 in MDTF cells was ∼35-fold (Fig. 1B), similar to that reported by using HeLa cells (32) and similar to the level of resistance to MLV conferred by Fv1 expression (34). Thus, TRIM5αrh,1 is fully active in murine MDTF cells. In CRFK cells, the degree of resistance to the HIV-1 vector conferred by TRIM5αrh,1 was slightly lower (∼10-fold) but similar to the degree of MLV resistance induced by Fv1 expression (Fig. 1C). A comparison of the various TRIM5α variants (Fig. 1D) revealed that neither human variant induced resistance to the HIV-1 vector in MDTF cells, whereas both rhesus variants strongly inhibited HIV-1 vector infection, consistent with previous studies (32). TRIM5αAGM,1 also conferred HIV-1 resistance, although it was slightly less active than the rhesus monkey variants (Fig. 1D).
Human TRIM5α Is Ref1. Most human cells behave superficially as if they carry the b-allele of Fv1, i.e., they exhibit specific resistance to N-MLV. TRIM5α<sub>human</sub> does not affect infection by standard NB-tropic MLV vectors (32), but we found that MDTF cells expressing TRIM5α<sub>human</sub> or TRIM5α<sub>murine</sub> were strongly resistant to N-MLV (Fig. 2A). Importantly, the same cells were only marginally less susceptible to B-MLV infection than unmodified control cells, and the level of N-MLV resistance conferred by TRIM5α<sub>human</sub> or Fv1 expression was comparable (Fig. 2A). Similar results were obtained in CRFK cells expressing TRIM5α<sub>human</sub> or TRIM5α<sub>murine</sub> (data not shown). We next tested whether TRIM5α<sub>human</sub> was necessary for N-MLV restriction in human cells. Two siRNA duplexes were chosen that efficiently silenced HA-TRIM5α human protein expression without cytotoxic effects, as evidenced by the fact that GFP expression by a cotransfected plasmid was unaffected (Fig. 2B). As can be seen in Fig. 2C and D, depletion of TRIM5α<sub>human</sub> in HeLa cells caused a 20-fold increase in N-MLV susceptibility but did not affect infection by B-MLV. Indeed, the levels of N-MLV infection approached those of B-MLV in TRIM5α-siRNA-transfected HeLa cells. Thus, TRIM5α<sub>human</sub> is sufficient to confer an N-MLV-specific restricting phenotype in otherwise nonrestricting cells (Fig. 2A) and is necessary for N-MLV restriction in human cells (Fig. 2C and D). As such, it appears responsible for the retrovirus restriction activity termed Ref1.

Non-Human Primate TRIM5α Variants Inhibit Infection by Widely Divergent Retroviruses. Previous studies implied that widely divergent retroviruses could be inhibited by the same saturable restriction factor (21, 35). Most notably, AGM cells apparently express a factor that restricts primate lentiviruses as well as N-MLV (21). In fact, TRIM5α<sub>AGM</sub> expression in MDTF cells conferred resistance to N-MLV (Fig. 3A). Thus, TRIM5α<sub>Duag1</sub> can indeed confer resistance to widely divergent retroviruses. Conversely, B-MLV infectivity was unaffected by TRIM5α<sub>AGM</sub> expression (Fig. 3B). TRIM5α<sub>human</sub> also specifically inhibited infection by N-MLV, albeit less efficiently than AGM TRIM5α (Fig. 3A and B). This result was surprising because previous studies did not reveal significant differences in N- and B-MLV titers on rhesus monkey fibroblasts (21). However, the earlier studies were done by using a different rhesus fibroblast cell line, and we therefore reexamined N-MLV restriction in FRIK4 cells (from which TRIM5α<sub>b1</sub> was derived). In fact, N-MLV titer was modestly reduced (~3-fold) as compared to that of B-MLV in FRIK4 cells (data not shown). It seems likely that TRIM5α<sub>b1</sub> overexpression accentuates restriction properties that are absent or marginal at physiological expression levels. Indeed, a similar phenomenon has been described for the b-allele of Fv1 (34). Nevertheless, the data in Figs. 1D and 3A establish that certain TRIM5α variants can restrict very divergent retroviruses.

Distinct Patterns of Sensitivity and Resistance to TRIM5α Variants Among Primate Lentiviruses. Next, we asked whether primate lentiviruses other than HIV-1 could be inhibited by TRIM5α. To allow studies using pseudotyped full-length reporter viruses
carrying GFP in place of Nef, we used TRIM5α-expressing CRFK cells as targets, because, unlike murine cells, feline cells can support Tat-dependent gene expression in the context of full-length reporter viruses. Previously, both HIV-1 and SIVMAC were found to be restricted in AGM CV-1 cells, whereas only HIV-1 is restricted in rhesus monkey cells, and neither is strongly restricted in human cells. These patterns of restriction were largely recapitulated in CRFK cells expressing TRIM5α/H9251AGM.1, TRIM5α/H9251rh.1, and TRIM5α/H9251hu.1 (Fig. 3B and C). Moreover, the transfer of the CA domain of SIVMAC into HIV-1 conferred resistance to TRIM5α/H9251AGM.1 as was reported in ref. 32, but not to TRIM5α/H9251AGM.3 (Fig. 3D). Thus, TRIM5α/H9251AGM.1 possesses broad antiretroviral activity. However, reporter viruses based on the genomes of SIVAGM Tan and SIVAGM Sab that are naturally found in AGMs (36, 37) were entirely resistant to TRIM5α/H9251AGM.1 (Fig. 3E and F). Nonetheless, these reporter viruses were partially sensitive to

Fig. 2. Human TRIM5α is necessary and sufficient to confer Ref1 activity. (A) Infection of MDTF cells that were unmodified (None) or expressing either of two human TRIM5α variants (hu.1 and hu.2) by N-MLV-GFP (filled bars) or B-MLV-GFP (open bars). The effects of Fv1α and Fv1β expression on N-MLV and B-MLV infection is shown for comparison. (B) Silencing of TRIM5α expression by using siRNA. HeLa cells were cotransfected with plasmids expressing HA-TRIM5α and GFP in the absence of siRNA (−) or in the presence of siRNAs targeting luciferase (Luc) or TRIM5α (lanes A and B). (C) Infection of human (HeLa) cells with N-MLV or B-MLV after transfection with either no siRNA, the control-luciferase-specific siRNA, or the TRIM5α-specific siRNA-A, as indicated. (D) Effects of control (Luc) and TRIM5α-specific siRNAs (A and B) on N-MLV (filled bars) and B-MLV (open bars) infection of HeLa cells.

Fig. 3. Non-human primate TRIM5α variants can inhibit infection by widely divergent retroviruses. (A) Infection of unmodified MDTF cells (None) or MDTF cells expressing AGM.1, rh.1, or rh.2 variants of TRIM5α by N-MLV (Left) and B-MLV (Right). (B–F) Infection of unmodified CRFK cells (None) or CRFK cells expressing hu.1, rh.1, or AGM.1 variants of TRIM5α, as indicated, by HIV-1 (B), SIVMAC (C), HIV-1(SIV CA) (D), SIVAGMTan (E), or SIVAGMSab (F).
enous TRIM5<sup>α</sup> was responsible for inhibiting EIAV infection in human cells, we depleted TRIM5<sup>α</sup> in HeLa cells by using siRNA. As can be seen in Fig. 4C, this depletion resulted in a significant (3-fold) increase in EIAV titer. Although the degree of increase in EIAV titer was less than that observed for N-MLV, this finding is consistent with previous observations, based on Ref<sub>1</sub> saturation (21), which showed that Ref<sub>1</sub> inhibits N-MLV more efficiently than EIAV.

**Discussion**

These studies show that CA-dependent retrovirus tropism for primate cells is governed in large part by species-specific variation in TRIM5<sup>α</sup>. In particular, we show that the restriction factor Ref<sub>1</sub>, which determines the differential sensitivity of human cells to N-tropic versus B-tropic MLV, is TRIM5<sub>α</sub><sub>hu</sub>. Indeed, these data demonstrate that the restriction factors previously termed L<sub>tv</sub> in primates and Ref<sub>1</sub> in humans are simply species-specific TRIM5<sup>α</sup> variants.

Unlike Fv<sub>1</sub>, which appears to be highly specific for MLV and incapable of inhibiting lentiviruses, TRIM5<sup>α</sup> from both humans and non-human primates restricted infection by widely divergent retroviruses, as was predicted by previous studies that used cross-saturation approaches (21). In particular, TRIM5<sub>α</sub><sub>AGM</sub> inhibited infection by HIV-1, SIV<sub>MAC</sub>, EIAV, and N-MLV. Given that the capsids of these retroviruses are highly divergent, this property is remarkable. It should be noted, however, that the general structures of retroviral capsids are quite well conserved, and all probably assemble based on similar hexameric lattices (38, 39). Nonetheless, it is intriguing that even though certain TRIM5<sup>α</sup> variants can inhibit very widely divergent retroviruses, the few amino acid changes that distinguish the B-MLV from the N-MLV constructs used in these studies can confer complete resistance to TRIM5<sup>α</sup>-mediated restriction.

TRIM5<sub>α</sub><sub>hu</sub> has the ability to inhibit infection by at least two divergent retroviruses, namely N-MLV and EIAV. Thus, in addition to potentially providing resistance to animal retrovirus infection, TRIM5<sub>α</sub><sub>hu</sub> may limit the usefulness of certain retroviruses, particularly EIAV, as vectors for gene therapy. TRIM5<sub>α</sub><sub>hu</sub> does not strongly inhibit any of the four primate lentiviruses tested. However, an important caveat is that the sample of primate lentiviruses tested was small and highly biased by the availability of full-length infectious clones and vectors. Because most primate lentiviruses have been passaged in human cells before cloning, they may be artificially selected for resistance to TRIM5<sub>α</sub><sub>hu</sub>. It will be interesting, and perhaps important, to determine whether other primate lentiviruses that threaten to cross species into humans are sensitive to TRIM5<sub>α</sub><sub>hu</sub> and other innate antiretroviral defenses, such as APOBEC3G.

The fact that TRIM5<sub>α</sub><sub>AGM</sub> was able to inhibit each of the three lentiviruses tested that do not naturally infect AGMs but did not inhibit two lentiviruses that are naturally found therein suggests that colonization of a particular species by a retrovirus may involve adaptation to avoid TRIM5<sup>α</sup>-mediated restriction.

One adaptation that appears unique to the HIV-1 lineage of primate lentiviruses and affects its restriction sensitivity in human and certain primate cells (35) is the propensity of its capsid to bind cyclophilin A (CypA). Whether the reduced infectivity of HIV-1 bearing CypA binding site mutations, specifically in human cells, is entirely due to TRIM5<sub>α</sub><sub>hu</sub> is unclear at present and is currently under investigation. Muta-

ions close to the CypA binding site and at other positions in CA also affect restriction in human and non-human primate cells (30, 31), and it will be interesting to determine how these affect TRIM5<sup>α</sup> sensitivity. Additionally, in cells from certain New World primates, specifically owl monkeys, CypA–capsid interaction is required for L<sub>tv</sub> restriction. Our preliminary findings, based on siRNA-mediated depletion, indicate that owl monkey TRIM5<sup>α</sup> is at least partially responsible for restriction in that
species (T.H. and D.P.-C., unpublished work), and it is therefore likely that CypA–capsid interactions modulate recognition by TRIM5α, although this is yet to be formally demonstrated.

Even though the sample size was small, we were easily able to document intraspecies TRIM5α sequence variation. Two TRIM5αthr variants were found in three human cell lines, and two TRIM5αIV variants were found in two rhesus monkey cell lines. Only one AGM cell line was included in this study, but previous observations (21) suggest that restriction factors are variable in this species. Taken together, these observations suggest that TRIM5α should be highly polymorphic both within and between species. Although we were not able to detect major differences in the restriction properties of TRIM5α variants in the two species where more than one variant was cloned, we almost certainly did not identify all variants. Differences that are too subtle to be recorded in the single-cycle infection assays used in this study might have a significant impact on the course of retroviral infections that last for several years and hundreds or thousands of virus replication cycles. Thus, the consequences of intraspecies TRIM5α sequence variation in the context of natural and experimental retroviral infections are unpredictable at present. Clearly, however, TRIM5α is a component of an important innate antiretroviral defense mechanism that is likely to have substantially affected the course of retroviral epidemics in humans and non-human primates.

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