Natural Infection of a Homozygous Δ24 CCR5 Red-capped Mangabey with an R2b-Tropic Simian Immunodeficiency Virus

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Summary

A homozygous 24-bp deletion (Δ24) was found in the CC chemokine receptor 5 (CCR5) of 11 out of 15 red-capped mangabeys (RCMs), *Cercocebus torquatus torquatus*, both in Africa and in an American zoo. The CCR5 Δ24 defect encompassed eight amino acids in frame in the fourth transmembrane region. Unexpectedly, RCM-009, one of 11 homozygotes (Δ24CCR5/Δ24CCR5), was found to be naturally infected with a divergent simian immunodeficiency virus (SIV) strain, which was not R5-tropic, but used CCR2b (R2b) as its major coreceptor. SIVrcmGab1 was the only R2b-tropic SIV among other divergent SIVs tested. Cells transfected with the Δ24 CCR5 did not support entry of R5-tropic SIVmac, SIVcpz, SIVmne, HIV-2, or HIV-1, and were also inactive in signal transduction mediated by β-chemokines. At 86.6%, the Δ24 allelic frequency was significantly higher than that of the 32-bp deletion found in humans. The Δ24 frequency was 4.1% in 34 sooty mangabeys (SMs), a geographically isolated subspecies that was naturally infected with R5-tropic SIV. Finding identical deletions in two mangabey subspecies separated for 10,000 years or more dates the Δ24 CCR5 deletion as ancient. However, the source of the selective pressure for the high rate of CCR5 deletion in RCMs remains to be determined. The high allelic frequency of the Δ24 CCR5 in RCMs, in comparison to that of SMs, suggests that R2b-tropism may have been acquired by SIVrcm, as an adaptation to CCR5 genetic defects appeared in its host.

Key words: SIVrcm • CCR5 • CCR2b • mangabey • Δ24 defect

After human immunodeficiency viruses types 1 (HIV-1) and 2 (HIV-2) were identified as causes of AIDS (1, 2), multiple non-human primate species in Africa were found to harbor their own divergent lentiviruses, the simian immunodeficiency virus (SIV)[1] group (3–9). Six simian species in Africa are known to be naturally infected, the sooty mangabey (SM), *Cercocebus torquatus atys*, in West Africa (6, 7); the four subspecies of African green monkeys (AGM), *Cercopithecus aethiops*, in East, Central, and West Africa (3, 10); the Sykes monkey, *Cercopithecus mitis*, in Kenya (8); the mandrill, *Mandrillus sphinx*, in Gabon (4); and the chimpanzee (CPZ), *Pan troglodytes*, in Gabon (5). We recently identified a new sixth lineage from a red-capped mangabey (RCM), *Cercocebus torquatus torquatus*, that was naturally infected in Gabon (9). Phylogenetic analysis of *pol* gene fragments showed an unexpectedly close relationship with the HIV-1/SIVcpz group of viruses, whereas analysis of *gag* gene sequences indicated a new lineage, independent from previously characterized SIVs (9). Additional sequence analysis of SIVrcm *gag* and *env* genes have also shown regions with relatively closer relationships with SIVagm and SIVsm (Gao, F., P.A. Marx, and B. Hahn, unpublished data). Therefore, SIVrcm appears to

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[1]*Abbreviations used in this paper:* AGM/agm, African green monkey; CPZ/cpz, chimpanzee; GFP, green fluorescent protein; MIP, macrophage inflammatory protein; RCM/rcm, red-capped mangabey; SIV, simian immunodeficiency virus; SM/sm, sooty mangabey.

have been generated by ancient recombinations involving ancestors of the sixth independent SIVrcm lineage and ancestors of the current HIV-1/SIVcpz and SIVagm groups. Because the SIV family is genetically divergent and naturally infects simians geographically isolated in Africa from thousands of years (3–10), SIV must have arisen in Africa long before the AIDS epidemic in humans and long before the earliest known HIV-1 infection in Africa (11). The ancient age of SIV offers the opportunity to experimentally study evolution of long-term, nonpathogenic virus–host relationships in comparison to the disease states found in the more recent human and macaque hosts (11, 12). Because of the unique phylogenetic features of SIVrcm, further characterization of its biological functions were done to understand its virus–host relationships.

The entry of HIV and SIV into host cells requires cell surface CD4 as a primary receptor to interact with the viral envelope (13). However, human CD4 molecules themselves, when expressed on non-human cell lines, do not render the cells susceptible to HIV-1 infection (14), suggesting that an additional cell surface receptor was required. Such coreceptors were identified on human CD4-bearing cells as belonging to the chemokine receptor family, all of which consists of surface seven-transmembrane G protein–coupled receptors (15–20).

Syncytia-inducing (SI) HIV strains that infect T cell lines are frequently found in late-stage HIV disease. These viruses generally use the chemokine receptor CXCR4 (15), the first chemokine coreceptor identified. In contrast, macrophage-tropic viruses that are non-syncytium-inducing (NSI), and may be found throughout the disease course, use CCR5 (16–20). CCR5 is the receptor for CC chemokines RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α and MIP-1β, all of which block cell entry of NSI strains of HIV-1 (21–23). CXCR4 is a receptor for the CXC chemokine SDF-1, which blocks entry of some SI HIV-1 strains (22, 23). Several HIV-1 isolates use multiple coreceptors CCR2b, CCR3, and CXCR4, in addition to CCR5 (19, 24). Unlike HIV-1, SIVs from monkeys are known to use the CC chemokine CCR5, but not CXCR4, for entry, regardless of their T cell or macrophage tropism (25–27). Additional coreceptors, GPR15, STRL33 and GPR1, mediate entry of some primary SIV isolates, probably accounting for SIV entry into CCR5-deleted PBMCs and T cell lines (25, 28–31).

HIV-1 CCR5-tropism is critical in HIV transmission and pathogenesis because homozygous $\Delta 32$ deletions were shown to confer resistance to infection against R5-tropic HIV-1 strains (32). Further molecular epidemiological studies showed that a homozygous defect in CCR5 was protective against HIV-1 transmission, whereas a heterozygous defect slightly delayed progression to AIDS (33–35). In contrast, similar defects and their role in protection against infection have not been documented in any non-human primate species.

In this study, we found that coreceptor usage by SIVrcmGab1 (9) was unique among divergent SIVs in that it used CCR2b but not CCR5 as a major coreceptor. To test for a genetic basis for this finding, we sequenced the CCR5 gene in a naturally infected RCM host. A homozygous 24-bp in-frame deletion ($\Delta 24$) in the fourth transmembrane region of CCR5 gene was found in the infected mangabey. The identical homozygous deletion was found in 11 out of 15 unrelated RCMs from widespread locations in Africa and the USA. The remaining four RCMs were heterozygotes. These results yielded a very high allelic frequency of 86.6%. The homozygous $\Delta 24$ CCR5 genotype did not support R5-tropic lentivirus infections and failed in signal transduction assays mediated by β-chemokines. The results show that a natural simian lentivirus infection can occur in the presence of CCR5 gene deletions without evidence of disease and that the mangabey deletion is ancient in comparison to the reported age of the CCR5 deletion in humans.

Materials and Methods

Virus Preparation. The preparation of virus stocks HIV-1 JR-FL, HIV-1 Z00979, HIV-2 A198411, HIV-2 A227011, SIVmac239, SIVcpzGab1, SIVmac1A11, and SIVagmTYO1 has been described previously (25, 31). SIVrcmGab1 was obtained from RCM-009, a naturally infected household pet in Gabon (9). HIV-2 A198411 and HIV-2 A227011 strains were provided by Dr. Beatrice Hahn (36) and were primary isolates from West Africa that were propagated and titered in human PBMCs in our laboratory as previously described (31). Luciferase reporter viruses were prepared by cotransfecting 293T cells with pNL-Luc-Env$^-$ or pSIV-Luc-R$^-$E$^-$ and vectors expressing different SIV or HIV-1 Envs, as previously described (16, 25).

Animals. All animal care and use was approved by the institutional animal care committees.

Tissue Culture. RCM PBMCs were prepared by Ficoll gradient separation, as previously described (7). Human osteosarcoma HOS.CD4 and glioma U87.CD4 cells, which stably express human and non-human primate chemokine receptors, have been described previously (25). GHOST.CD4 cl.34 cells are human osteosarcoma cells expressing human CD4 and containing the green fluorescence protein (GFP) gene controlled by the HIV-2 LTR promoter as the indicator of lentivirus infection. GHOST.CD4.Hu-BOB and GHOST.CD4.Hu-Bonzo cells, which express human coreceptors BOB (GPR-15) and Bonzo (STRL33), were provided by V.N. KewalRamani of New York University Medical Center.

Cell Separation from Feces. Fecal specimens from free-living mangabeys in Gabon were collected fresh by spreading plastic sheets under a tree being used for sleeping by a wild mangabey troop. The mangabeys were observed through binoculars in late afternoon, to verify the species of the individual depositing the specimen. The specimen was collected immediately after it dropped to the plastic. In the field, 35 ml PBS was added to 10 g of fresh feces and suspended by shaking and vortexing. The fecal suspension was layered onto 15 ml of lymphocyte separation medium (Organon-Teknika, Durham, NC). Using a portable centrifuge, the mixture was spun at 3,000 rpm for 20 min. Cells were harvested at interface, in a volume of ~15 ml. If the cell layer was diffuse after a single centrifugation, medium above and below the interface was collected. The ficoll separation step was repeated up to five times, using 3.0 ml of lymphocyte separation

medium, until the cell layer was clearly defined. Cells were suspended in 10 ml PBS, washed twice, and stored in a portable liquid nitrogen tank.

Analysis of Genomic DNA and Cellular RNA for CCR5 Deletion. The full length RCM-CCR5 was amplified as previously reported (25) for rhesus CCR5, using primers that flank the entire CCR5 coding region. PCR primers that hybridized 5′ and 3′ to a *ccr5* region flanking the Δ24 deletion were as follows: 5′ primer (nucleotides 318–343 in the CCR5 coding region): 5′-GCT CTA TTT TAT AGG CTT CTT CTC TG-3′, and 3′ primer (510–530): 5′-GTG TAA TGA AGA CCT TCT CTC TGA GAT CTG-3′. Using this primer pair, 189 or 213 bp *ccr5*-specific fragments with and without the 24-bp deletion were amplified from genomic DNA (0.5 μg). The PCR reaction was according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN) in 100 μl containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl$_2$, 0.1% Triton X-100, 200 μM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of each primer, and 0.5 U of DNA Taq polymerase (Boehringer Mannheim). Amplification cycles were 95°C for 2 min followed by 35 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s plus the last extension of 72°C for 10 min. RNA was extracted from cells using Triazol (GIBCO BRL, Gaithersburg, MD) and treated with 10 U RNase-free DNase (Promega Corp., Madison, WI). cDNA was prepared from 5 μg RNA using an oligo-dT primer Superscript reverse transcriptase (GIBCO BRL), and resuspended in 80-μl 10 mM Tris, pH 8.0, 1 mM EDTA (TE). The cDNA was amplified over 35 cycles using the primer pair specific for the *ccr5* fragment flanking the Δ24 deletion as described above.

Plasmid Construction and Expression of RCM-CCR5. The procedure for constructing expression plasmids was the same as that described for rhesus CCR5 and CXCR4 (25). In brief, virus stocks for the retroviral vectors expressing CCR5 and CXCR4, pBabe/CCR5, and pBabe/CXCR4, respectively, were prepared by cotransfecting 293T cells with the murine leukemia virus (MuLV)-gag/pol vectors pSV-psi-MLV-env⁻ and pcVSV-G. Virus was harvested 48 h after transfection and frozen at −70°C. 293, HOS.CD4, and U87.CD4 cells were infected at the same time with 2 ml of the pBabe virus and were selected 48 h later in culture medium containing 1.0 μg/ml puromycin (Sigma Chemical Co., St. Louis, MO). Puromycin-resistant cells were then used for HIV-1 and SIV infection with replication-competent or luciferase reporter viruses.

Virus Entry Assay. To test for viral entry, 3×10^4 cells were seeded in 24-well plates in culture medium and infected with luciferase reporter virus (20 ng p24 or p27) in a total volume of 300 μl containing 1 ng/ml polybrene. After incubation overnight, 1 ml of medium was replaced in each well. After three days at 37°C, 100-μl lysates were prepared and luciferase activity in 20 μl was measured using commercially available reagents (Promega Corp.). For GFP assay, 2×10^4 GHOST cells expressing coreceptors were seeded into 24-well plates. After 24 h, 5 ng p27 of each SIV was inoculated for 12 h with 20 μg/ml polybrene. Input virus was removed by washing, and cells were maintained in DMEM medium with 10% FCS. GFP expression was detected by FACS® analysis (Becton Dickinson, San Jose, CA) 48 h after infection.

Viral Infectivity Assays. To determine coreceptor usage of different viruses, 2×10^4 HOS.CD4 or U87.CD4 cells stably expressing human or rhesus coreceptors were seeded in 24-well plates. The next day the adherent cells were washed with PBS and infected with 5 ng p27 of SIV or 500 TCID$_{50}$ of HIV-1. After 12 h, virus was removed by replacing the medium three times. The cells were maintained in medium with 2% FCS for up to 10 d.

Virus production was measured by harvesting aliquots of each supernatant and measuring p27 (Coulter, Chicago, IL) or p24 (Abbot, Chicago, IL) by ELISA. After removing 0.5 ml of culture supernatant per well for assay, the same amount of fresh medium was added to the culture.

Calcium Ion Influx Assay Using Flow Cytometry. The assay was performed as previously described (37). In brief, 293 cells expressing RCM-CCR5 or RCM-Δ24-CCR5 were harvested from tissue culture plates using a cell scraper. The cells were washed with tissue culture medium before resuspending them at 10^6 cells/ml in HBSS containing 0.5% (wt/vol) BSA, 4 mM probenicid (Sigma Chemical Co.) and 4 μg/ml Fluo-3 AM, 10 μg/ml Fura-Red, and 0.02% Plurionic F127 (all from Molecular Probes, Eugene, OR). The cells were incubated at 37°C for 30 min then centrifuged at 1,500 rpm for 5 min before being gently resuspended at 10^6 cells/ml HBSS containing 0.5% BSA. The cells were left at room temperature for 20 min. All analyses were performed using a FACSCalibur® flow cytometer with Cellquest software (Becton Dickinson). Equal amounts of the cells were removed, further diluted to 10^6 per ml in HBSS containing 0.5% BSA and warmed to 37°C for 5 min. The sample temperature was maintained throughout the assay by a water jacket at 37°C. Data were collected over a 3-min period 20 s after adding 200 nM β-chemokines, MIP-1β, or RANTES. A ratio of Fluo-3/Fura-red fluorescence was calculated using FACS Assistant software (Ray Hicks, Cambridge University, Cambridge, UK). Data are displayed as a density plot of time verses fluorescence ratio and also as a line graph of time versus mean fluorescence ratio, which was calculated using FACS Assistant Software.

Sequence Analysis. The complete sequence of RCM-CCR5 was obtained using an automated sequencer (ABI Prism 377 DNA Sequencer; PE Applied Biosystems, Foster City, CA). At least two molecular clones were sequenced from two animals using overlapping primers. The RCM-CCR5 sequence are available from EMBL/GenBank/DDBJ under accession numbers AF084002–AF084004 and AF094752–AF094753.

Results

Coreceptor Usage of SIVrcmGab1. SIVsm, SIVagm, and SIVcpz are R5-tropic, divergent SIVs found in East, West, and Central Africa (25–27, 30, 31). Because all naturally occurring SIVs described thus far are R5-tropic, the coreceptor use of the new virus, SIVrcmGab1, was first tested on GHOST cells expressing CD4 plus CCR5 or CXCR4 derived from humans and macaques. Unexpectedly, no entry was detected (data not shown). Known HIV-1 and SIV strains were included to demonstrate the appropriate coreceptor function of each cell-type. To further examine SIVrcm coreceptors, human chemokine receptors CCR1, CCR2b, CCR3, CCR4, CCR5, CCR8, CXCR4, STRL33 (Bonzo), and GPR15 (BOB) were tested in the GHOST cell assay (Fig. 1). Among the SIV and HIV strains tested, only SIVrcm entered CCR2b-expressing cells (Fig. 1). This entry was blocked by anti-CD4 antibody Leu-3a (data not shown), indicating CD4 dependency. SIVmac, SIVagm, HIV-1JR-FL, and HIV-1ADA, primate lentiviruses of known coreceptor useage, were included to demonstrate appropriate activity of the cell lines (Fig. 1). SIVrcm also used STRL33 (Bonzo), but with ~10-fold
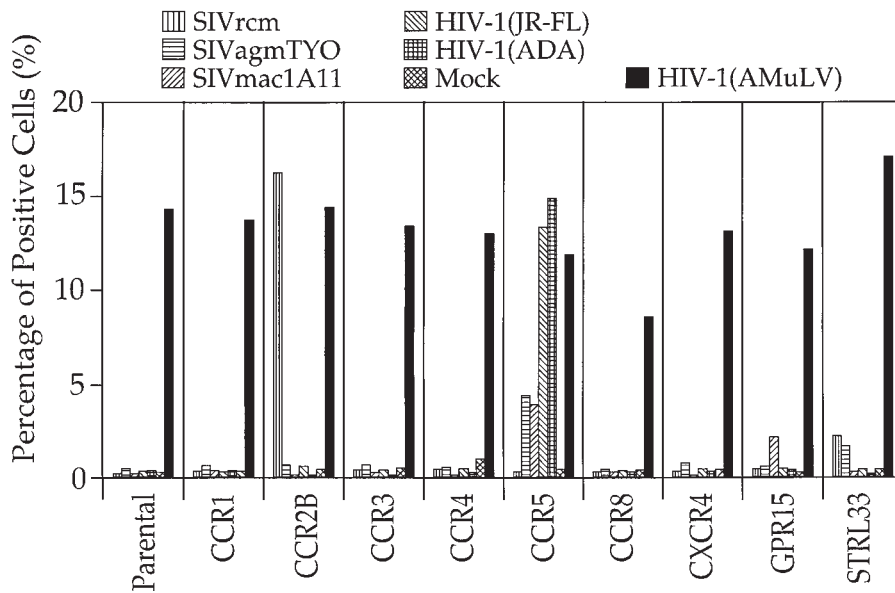
Figure 1. Entry of primate lentiviruses into GHOST cells expressing nine chemokine receptors. HIV-1 pseudotyped viruses contained R5-tropic envelopes from HIV-1 JR-FL and HIV-1 ADA or amphotropic murine leukemia virus (*AMuLV*) envelope. Infectious SIV strains were SIVmac1a11, SIVagmTYO, and SIVrcmGab1. Negative control cells were parental GHOST cells.

less efficiency. However, low STRL33 expression could not be excluded as the cause of inefficient STRL33 use, because specific antibodies for testing STRL33 cell surface expression are unavailable. The remaining chemokine receptors, including GPR-15 (BOB), were not used by SIVrcm.

Sequence Analysis of RCM-CCR5. To understand why SIVrcm was uniquely R2b-tropic, but not R5-tropic, among widely divergent HIVs and SIVs, full-length RCM-CCR5 was amplified from the DNA of SIV-infected RCM-009 and completely sequenced as previously described (25). The RCM-009 CCR5 sequence was aligned and compared with the SM consensus amino acid sequence (31), which had been derived from four SMs naturally infected with R5-tropic SIVsm in West Africa (31, 38). The RCM sequence differed from SIVsm-infected SM CCR5 genes at only three amino acid positions, I42V, L146V and P179S. However, a homozygous 24-bp deletion in the CCR5 was identified from the SIV infected RCM-009 (Fig. 2). The deletion encompassed 8 amino acids in frame in the fourth putative transmembrane region, upstream from the Δ32 deletion found in the second extracellular loop of human CCR5 (32). To test for spurious PCR deletion artifacts, five additional CCR5 clones from RCM-009 were sequenced and had the identical deletion (data not shown). Moreover, a limiting dilution of the original genomic DNA of RCM-009 showed that all 20 end point PCR products gave the identical deletion by direct sequencing analysis, ruling out PCR artifacts (data not shown). Identical results were also obtained using a total of three pairs of PCR primers flanking the deletion region. The identical homozygous deletion was also identified from 10 additional RCMs (some are shown in Fig. 2) using the above technique. Since limiting dilution allowed 20 separate amplifications of single molecules, artifacts between DNA products were avoided.

This deletion was not present in the CCR5 consensus sequence obtained from four SMs (Fig. 2) naturally infected in West Africa with four distinct R5 tropic viruses, SIVsmSL92a, -c, -d, and -e (38). These results show that two closely related but geographically isolated mangabey sub-species were naturally infected with R5- or R2b-tropic SIVs that correlated with homozygous full-length or deleted CCR5 genes, respectively. The finding of an R2b-tropic SIVrcm in a natural host having a homozygous CCR5 defect suggested that SIV R2b tropism and defective CCR5 genes may be a related phenomenon.

Comparison of the Allelic Frequency of Δ24 CCR5 among Geographically Isolated RCMs and SMs. To analyze the allelic frequency of Δ24 deletions, genomic DNA was extracted from 14 additional RCMs from widely separate locations in Gabon and an American zoo colony. DNA was analyzed from a total of six zoo-housed RCMs in America, from eight household pets in Gabon, and from one fecal sample collected fresh from a wild, free-living mangabey, RCM-F3. PBMCs from the zoo mangabeys in the USA and all household pets, except for RCM-009, were negative for SIVrcm antibodies by Western blot or culture and PCR as previously described (9). RCM-CCR5 deletions were detected using primers that specifically amplified a 213-bp fragment spanning the Δ24 region (Fig. 2). Four zoo RCMs were homozygous Δ24 and two were heterozygous (Fig. 2). Fig. 2 shows the sequence of the Δ24 and wild-type DNA fragments from the zoo heterozygote, RCM-1208, and two homozygotes from the zoo, RCM-997 and -1049. Of the remaining seven household pets in Gabon, five were homozygous for Δ24 CCR5 and two were heterozygotes. RCM-009, the infected mangabey, was homozygous for the CCR5 deletion (Fig. 2). No RCMs on either continent were homozygous wild-type for CCR5. Fecal DNA from RCM-F3, a wild, free-living RCM, was also homozygous Δ24 CCR5 (data not shown).
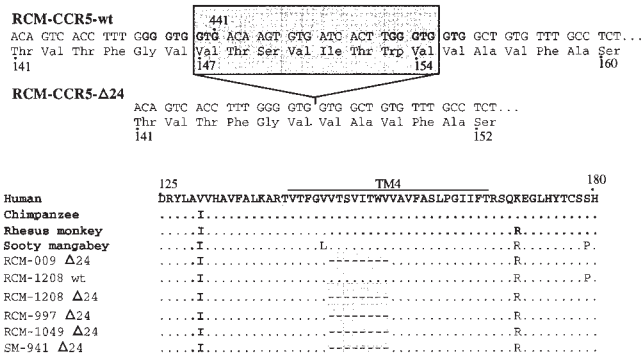
RCM-CCR5-wt

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          441
ACA GTC ACC TTT GGG GTG GTG ACA AGT GTG ATC ACT TGG GTG GTG GCT GTG TTT GCC TCT...
Thr Val Thr Phe Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser
141                  147                      154                              160
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RCM-CCR5-Δ24

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ACA GTC ACC TTT GGG GTG GTG GCT GTG TTT GCC TCT...
Thr Val Thr Phe Gly Val Val Ala Val Phe Ala Ser
141                                          152
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               125                      TM4              180
Human          DRYLAVVHAVFALKARTVTFGVVTSVITWVVAVFASLPGIIFTRSQKEGLHYTCSSH
Chimpanzee     .....I...................................................
Rhesus monkey  .....I..........................................R........
Sooty mangabey .....I.............L............................R.......P.
RCM-009  Δ24   .....I.................--------.................R........
RCM-1208 wt    .....I..........................................R.......P.
RCM-1208 Δ24   .....I.................--------.................R........
RCM-997  Δ24   .....I.................--------.................R........
RCM-1049 Δ24   .....I.................--------.................R........
SM-941   Δ24   .....I.................--------.................R........
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Figure 2. Alignment of CCR5 DNA fragments encompassing the Δ24 region of RCMs and other primates. CCR5 fragments were derived by PCR of DNA from RCM-009, an SIVrcm-infected household pet in Gabon, and American zoo animals 997, 1049, and 1208. SM-Δ24-CCR5 (SM-941) was from an SM housed at the Tulane Regional Primate Research Center. RCM-CCR5 sequences were compared with consensus human, CPZ, rhesus monkey, and SM (25, 31). The GGGTGGTG recombination sequences are in bold. Amino acid comparisons are shown in single capital letters; gray box shows amino acids in the 24-bp deletion.

To calculate an allelic frequency for Δ24, pedigree charts from zoo animals were examined to determine if in-breeding had occurred. Six zoo animals were unrelated. A seventh was a full sibling and was therefore excluded from the analysis. The pedigree charts also showed that the American and African groups could not be related, because of age and birthplace. The household pet RCMs were also unrelated because they were wild-caught by their owners at different locations and kept alone with no known contact with each other. The allelic frequency for Δ24 CCR5 for the 15 RCMs on two continents was 86.6% (Table 1). In marked contrast, none of 34 African pet or wild SMs were homozygous for the Δ24, but 3 were found to be heterozygous, giving an allelic frequency of 4.1%, >20-fold lower than RCMs ($P < 0.001$). All African SMs were also unrelated because they were young, wild-caught household pets from different parts of two West African countries, Liberia and Sierra Leone. Fig. 2 shows the Δ24 deletion found in an SIVsm-infected captive, SM-941, at the Tulane Regional Primate Research Center. The data showed a correlation between high wild-type and deleted CCR5 allelic frequencies with R5- and R2b-tropic viral infection.

CCR5 Genotyping of Primate Species. Taking the importance of the Δ32 defect to HIV transmission and AIDS progression in humans into consideration, we screened a Caucasian cohort for the Δ24 CCR5 genotype using the CCR5-specific PCR method that was specific for the deletion as described in Materials and Methods and shown in Fig. 2. In a total of 409 individuals tested, 9 were homozygous for Δ32, 41 were heterozygous for Δ32, and the remaining 306 were wild-type CCR5. The allelic frequency was 7.3% (Table 1). All of the human samples were negative for the 189-bp fragment that is diagnostic for this deletion (Table 1). Therefore, the Δ24 genotype identified in RCMs must be rare or absent in Caucasian populations. However, other human populations must be tested. In addition, we screened nine CPZs, 34 SMs, 7 lunulatus mangabeys, and 35 rhesus monkeys. Besides the three Δ24 heterozygous SMs described above, the other non-human primates tested had neither Δ24 nor Δ32 CCR5 genotypes. However, larger numbers of each simian species must be tested.

Functional Analysis of RCM-CCR5. To test function of RCM-CCR5 genes, signal transduction was tested by a Ca^{2+} influx assay as previously described (37). Methods used for stable transfection of 293 cells with Δ24 or full

Table 1. *Distribution of Δ32 and Δ24 CCR5 Genes in Humans and Non-human Primates*

Primates	Wild-type	Δ24/Δ24	Δ24/wt[‡]	Δ32/Δ32	Δ32/wt	Total Δ32 bp or Δ24 bp (allelic frequency)
Red-capped Mangabey						
(*Cercocebus torquatus torquatus*)	0	11	4	0	0	15 (86.6%)
Sooty Mangabey						
(*Cercocebus torquatus atys*)	31	0	3	0	0	34 (4.1%)
Caucasian Cohort	356	0	0	9	41	406 (7.3%)
						(0–13.7%)*
White-crowned Mangabey						
(*Cercocebus torquatus lunulatus*)	7	0	0	0	0	7
Chimpanzee						
(*Pan troglodytes*)	9	0	0	0	0	9
Rhesus Monkey						
(*Macaca mulatta*)	35	0	0	0	0	35

*The range reported for different geographical human groups (40).

[‡] wt, wild-type.

length RCM-CCR5 were reported (25). Quantitative CCR5 gene expression was determined by measuring intracellular levels of mRNA transcripts by reverse transcription PCR assay (Fig. 3, *A* and *B*). Full length and deleted transcripts were expressed at similar levels. MIP-1β was used to induce signal transduction, because it is specific for CCR5. MIP-1β induced Ca^{2+} influx in 293T cells transfected with RCM-wt-CCR5 (Fig. 3 *C, right*) but not in RCM-Δ24-CCR5 transfected cells (Fig. 3 *C, left*). Assays using RANTES gave similar results (data not shown). Therefore, RCM-Δ24-CCR5 has lost physiologic function for signaling, like the Δ32 CCR5 human allele (32).

The coreceptor function of RCM Δ24 and wild-type CCR5 genes, obtained from the zoo heterozygote, were further tested in transfected HOS.CD4 cells. The RCM-CCR5–transfected HOS.CD4 cells were inoculated with single-cycle HIV-1– or SIVmac-based luciferase reporter viruses pseudotyped by various envelopes as previously reported (25). When equal amounts of viruses were added, no viral entry by any of eight R5-tropic viral envelopes was detected in Δ24-RCM-CCR5 cells (Fig. 4 *A*). However coreceptor function of RCM-wt-CCR5 was intact for each R5-tropic viral envelope (Fig. 4 *A*).

To determine if SIVrcm could replicate in cells expressing the homologous full-length CCR5 derived from its own host species, SIVrcm was inoculated on HOS.CD4 cells expressing RCM-wt-CCR5 (Fig. 4). Replication assays were used because SIVrcm *env* clones are not yet available for the reporter assay. All six R5-tropic viruses, including HIV-1, HIV-2, and highly divergent strains of SIV,
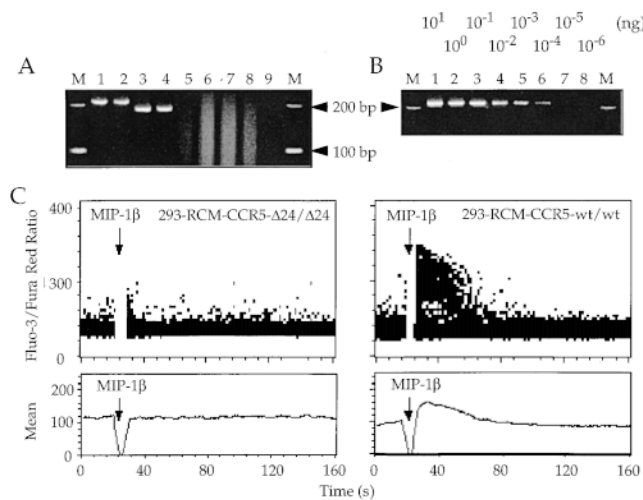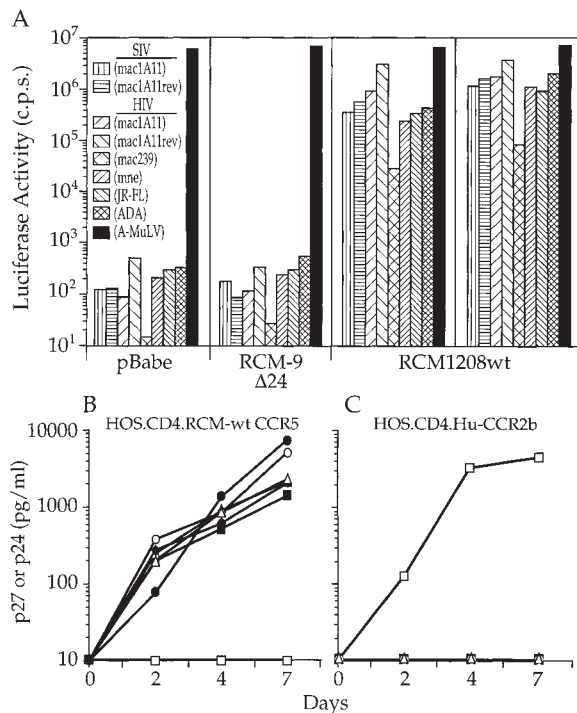

Figure 4. (*A*) Entry of luciferase reporter viruses into cells expressing RCM-wt-CCR5 or RCM-Δ24-CCR5. Hos.CD4 cells were permanently transfected with the pBabe vector alone, wt CCR5, or Δ24 CCR5. Two separate cell lines are shown for RCM-1208. Envelopes in brackets are from a panel of R5-tropic lentiviruses. HIV-(A-MuLV) contains an amphotropic murine leukemia virus envelope and was used as a positive control for viral entry and synthesis. (*B*) Growth of SIV and HIV in Hos.CD4 cells transfected with RCM-wt-CCR5 or CCR2b derived from humans. R5 tropic viruses were as follows: ■, HIV-1 (JR-FL); ●, HIV-1Z00979; ▲, HIV-2 A198411; ◆, HIV-2 A227011; ○, SIVmac239; and Δ, SIVcpzGab1. SIVrcmGab1 (□) did not infect cells expressing wild-type CCR5 from its own host species (*B*). (*C*) SIVrcmGab1 was the only lentivirus that replicated in CCR2b-expressing cells.

Figure 3. Calcium influx in 293 cells transfected with RCM-wt-CCR5 and RCM-Δ24-CCR5. *A* shows 213-bp DNA fragments derived from cells transfected with the wild-type CCR5 allele of RCM 1208 (lanes *1* and *2*) and 189-bp fragments from the deleted allele of RCM-1208 (lane *3*) and -009 (lane *4*). Lanes *5–8* show the same PCR reactions without the reverse transcription step. Lanes *9* and *M* are buffer alone and molecular weight markers, respectively. *B* shows quantitative PCR of the 213-bp fragment by end-point dilution as standards for comparing quantities of transcripts in cells in *A*. (*C*) Treatment with 200 nM MIP-1β resulted in a peak of Ca^{2+} influx in cells with RCM-wt-CCR5 (*right*) but not in cells containing RCM-Δ24-CCR5 mRNA (*left*).

replicated in cells expressing RCM-wt-CCR5 (Fig. 4 *B*). However, SIVrcm did not replicate in RCM-wt-CCR5 cells (Fig. 4 *B*). In contrast, SIVrcm was the only lentivirus out of seven tested that replicated in cells transfected with CCR2b (Fig. 4 *C*). Similar results were obtained using U87.CD4.Hu-CCR2b. Therefore, SIVrcm could not use its own full-length, functional CCR5.

Discussion

In this study we describe a natural SIV infection of an RCM that was homozygous for a deletion in the CCR5 gene. The naturally occurring SIVrcm was found to be R2b-tropic and not capable of using full-length human or RCM-CCR5. Sequencing of the RCM-CCR5 revealed a unique 24-bp deletion in the fourth transmembrane region. Characterization of the Δ24 CCR5 allele showed that it was common in both wild-caught RCMs in Gabon and in unrelated captives in an American zoo. The overall Δ24 allelic frequency was 86.6%, significantly higher than the

4.1% frequency found in SMs, a closely related species from West Africa that was naturally infected with R5-tropic SIVs (31). The wild-type and deleted CCR5 alleles were cloned from a heterozygous RCM, because no homozygotes for wild-type CCR5 were found. Expression in human cell lines showed that only the full-length wild-type RCM-CCR5 was functional as a coreceptor for R5-tropic HIV-1, HIV-2, SIVsm, and SIVmac strains. The full length wild-type RCM-CCR5 allele was also capable of signaling when tested for MIP-1β–induced calcium influx. Although Δ24 CCR5 mRNA was expressed in transfected cells, the cells were inactive in signal transduction.

Since the 24-bp deletion encompasses eight amino acids in frame, the deleted gene may be synthesized as a truncated protein inside the cell. Stable cell surface expression of the truncated protein is possible, but appears unlikely because signal transduction was not detected (Fig. 3). The deletion may adversely affect expression because it shortens the membrane-spanning domain (Fig. 2) from 26 amino acids to ~19 amino acids. However, expression of Δ24 CCR5 with limited function is possible because the hydrophylic amino acids (R140 and R168) that determine fourth transmembrane region properties were preserved. Benkirane et al. (39) reported that the 32-bp deletion in the region of the second extracellular loop of the human CCR5 gene resulted in the loss of phosphorylation of the CCR5 gene product. The truncated CCR5 protein remained in the endoplasmic reticulum and was not transported. Because the 24-bp deletion reported here is in-frame, and in the fourth transmembrane domain, it is not possible to predict whether or not the mutation's effect will be similar to that observed by Benkirane et al. for the Δ32 deletion. Additional studies are needed to characterize the 24-bp defect and its effect on function, expression, and transport.

Although only 15 RCMs were tested, the data support a naturally high frequency for the deleted allele. The high frequency was found in two groups of RCMs on different continents, a finding that could not be explained by inbreeding. Furthermore, a high frequency of deleted genes was also present in Gabonese household pets and in one wild RCM. All Gabonese mangabey alleles were derived from wild, free-living populations from different areas of the country. In marked contrast, 34 household pet and wild SMs from Sierra Leone and Liberia showed a significantly lower frequency (4.1%) similar to the prevalence of the Δ32 CCR5 found in certain human populations (40). This cause for the high frequency in RCMs is unknown, but may have been caused by the spread of a small founder population of RCMs. Comparative genetic studies between SMs and RCMs could be used to determine if there was a bottle neck effect on the RCM sub-species that produced the high frequency of deleted CCR5 genes and a unique SIV–host relationship.

Hypotheses have been proposed that the appearance of the human CCR5 deletion was in response to a past epidemic in which the loss of the CCR5 gene conferred resistance against an epidemic agent (40). Similar forces may have been involved in the selection of the mangabey defect. The deletions in mangabeys and humans may represent convergent evolution, due to similar ecological pressures. However, their temporal occurrence and geographical distributions appear to be highly discordant, based on results thus far. The historic ranges and dispersals of SMs and RCMs containing the Δ24 CCR5 deletion indicate that this genetic defect arose some time before mangabey subspeciation. Subspeciation of these coastal mangabeys developed more than 10,000 yr ago due to natural barriers that formed in their range. The Dahomey gap, which is a 900-km Savannah break in upper and lower guinea forest, is one such barrier. Rivers in the Dahomey gap may also have been barriers that contributed to development of the subspecies (41). This date is significantly older than the 700 yr estimate for the age of the 32-bp CCR5 deletion in humans (40). Why the mangabey CCR5 deletion would be much older than the human deletion remains to be determined. The difference in ages of the two defects suggest that the selective pressures for the deletions were different or were caused by similar events widely separated in time.

The mangabey and human CCR5 deletions are also discordant in geographical distribution. The human Δ32 is most common in Northern Europeans and is absent among populations native to Africa (40). In marked contrast, the mangabey CCR5 deletion was found in equatorial African mangabeys. The discordance in Δ24 CCR5 distributions further indicates that the selective pressures for the emergence of the mutations occurred under quite different circumstances.

Other primate species, including macaques, CPZs, and humans, have the same possible recombination site, GGG-TGGTG (Fig. 2), but the deletion was not found in >400 individuals tested from these three species (Table 1). More monkeys, as well as more humans, must be tested to extend these results. The finding of a deleted CCR5 allele in a more readily available research monkey species such as the macaque would be useful for pathogenic studies.

This study also raises questions about the origin of R2b tropism in SIV. This tropism is unknown among highly divergent SIVsm, SIVmac, SIVagm, and SIVcpz, all of which are R5-tropic (25–28, 30, 31). R5-tropic SIV strains originated from different parts of Africa, showing their widespread distribution among naturally infected simian species. Therefore, it is possible that SIVrcm was derived from the more common R5-tropic SIV and switched its tropism to CCR2-based coreceptors in response to the appearance of deleted CCR5 genes at high frequencies in its host. SIVrcm R2b-tropism may have arisen when RCMs became a separate, isolated subspecies in relatively ancient times.

Adaptation of SIV from CCR5 to CCR2b would require an intermediate coreceptor. Such a coreceptor is available for SIVrcm, based on in vitro studies that showed that the virus used STRL-33 (Fig. 1). Because SIVrcm and all other SIVs are dual tropic (Fig. 1 and references 25, 28, 30, 31), R5-tropic ancestors of SIVrcm may have replicated in mangabey hosts using a minor coreceptor and then adapted to CCR2-based coreceptors, the closest chemo-

kine coreceptor to CCR5 (42, 43). This hypothesis can be tested by infecting homozygous deleted mangabeys with R5-tropic SIV. If selective pressure can induce coreceptor switches, then the selection pressure from CCR5-directed drug therapy may cause viral adaptation to other coreceptors resulting in drug resistance. This concern is raised because it has been shown that certain HIV-1 strains used CCR2b as a coreceptor, and changes in coreceptor tropism are known to occur in HIV-1–infected people (42–44).

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