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Homozygous Defect in HIV-1 Coreceptor Accounts for Resistance of Some Multiply-Exposed Individuals to HIV-1 Infection

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Summary

Rare individuals have been multiply exposed to HIV-1 but remain uninfected. The CD4$^+$ T-cells of two of these individuals, designated EU2 and EU3, are highly resistant in vitro to the entry of primary macrophage-tropic virus but are readily infectable with transformed T-cell line adapted viruses. We report here on the genetic basis of this resistance. We found that EU2 and EU3 have a homozygous defect in *CKR-5*, the gene encoding the recently described coreceptor for primary HIV-1 isolates. These individuals appear to have inherited a defective *CKR-5* allele that contains an internal 32 base pair deletion. The encoded protein is severely truncated and cannot be detected at the cell surface. Surprisingly, this defect has no obvious phenotype in the affected individuals. Thus, a *CKR-5* allele present in the human population appears to protect homozygous individuals from sexual transmission of HIV-1. Heterozygous individuals are quite common (~20%) in some populations. These findings indicate the importance of *CKR-5* in HIV-1 transmission and suggest that targeting the HIV-1–CKR-5 interaction may provide a means of preventing or slowing disease progression.

Introduction

The vast majority of people are susceptible to infection with HIV-1. However, rare individuals have been described that appear to remain uninfected by HIV-1 despite histories of multiple high-risk sexual exposures to the virus (Clerici et al., 1992; Langlade-Demoyen et al., 1994; Rowland-Jones et al., 1995; Paxton et al., 1996). While in some cases this may simply be stochastic or may be due to an extremely quiescent infection, Paxton et al. (1996) have shown that the CD4$^+$ T-cells of some of these individuals resist high doses of virus in vitro. Of 25 exposed–uninfected (EU) individuals studied, the CD4$^+$ T-cells (Paxton et al., 1996) and macrophages (R.I. Connor et al., submitted) of two such individuals, designated EU2 and EU3, required about 1000-fold more virus to establish infection than contol cells from unexposed donors. While a small fraction of the cells did become infected with this high inoculum, the virus failed to replicate further. Analysis of the early events of the viral replication cycle showed that macrophage-tropic HIV-1 isolates failed to enter or fuse to the CD4$^+$ cells of these two individuals (Dragic et al., 1996). Thus, the resistance of these individuals to sexual transmission of HIV-1 was likely to have resulted from the inability of their cells to support entry of macrophage-tropic virus.

HIV-1 can broadly be divided into macrophage- or T-tropic isolates (Gartner et al., 1986; Koyanagi et al., 1987; Fisher et al., 1988). Macrophage-tropic nonsyncytium-inducing (NSI) isolates infect primary macrophages but fail to infect transformed T-cell lines, while T-tropic syncytium-inducing (SI) strains have the reciprocal tropism. Both classes of HIV-1 efficiently infect CD4$^+$ T-cells isolated from peripheral blood mononuclear cells (PBMC). Macrophage-tropic NSI viruses appear to be preferentially transmitted by sexual contact and constitute the vast majority of virus present in newly infected individuals (Zhu et al., 1993). The T-tropic SI viruses generally appear late in the course of infection during the so called "phenotypic switch" that often precedes the onset of AIDS symptoms (Schuitemaker et al., 1992; Connor and Ho, 1994).

HIV-1 replication is initiated by attachment of the virus to the cell surface via high affinity binding of the envelope glycoprotein (Env) to CD4 on the cell surface (reviewed in Sattentau and Weiss, 1988). Subsequently, the viral envelope fuses to the cell membrane, depositing the viral core in the cytoplasm. The fusion reaction is mediated by newly described seven transmembrane domain G protein–coupled molecules termed coreceptors (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). The molecular basis of HIV-1 tropism appears to lie in the ability of Envs from macrophage-tropic and T-tropic viruses to interact with different coreceptors. T-tropic viruses tend to use Fusin, a previously identified seven transmembrane protein related to the IL-8 receptor (Feng et al., 1996). Macrophage-tropic viruses primarily use CKR-5 (for C-C chemokine receptor-5), a newly described seven transmembrane domain chemokine receptor (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Use of other chemokine receptors such as CKR-2B and CKR-3 by a minority of viruses has also been reported (Choe et al., 1996; Doranz et al., 1996).

Physiologically, chemokine receptors mediate the chemotaxis of T-cells and phagocytic cells to areas of inflammation (reviewed by Horuk, 1994). Upon ligand binding, the receptors transduce an intracellular signal that results in the rapid mobilization of intracellular calcium. Each of the eight known chemokine receptors is a G protein–coupled seven transmembrane domain protein with a characteristic pattern of ligand binding (reviewed by Schall, 1991). CKR-5, which also serves as a major coreceptor for macrophage-tropic HIV-1,

binds the β-chemokines RANTES (regulated on activation, normal T expressed and secreted), MIP-1α (macrophage inflammatory protein) and MIP-1β (Samson et al., 1996). The ligand for Fusin has not yet been identified. High levels of RANTES, MIP-1α, or MIP-1β prevent replication of macrophage-tropic, but not T-tropic strains of HIV-1 (Cocchi et al., 1996). This inhibition is due to the binding of chemokines to CKR-5, resulting in a block to viral entry and fusion (Deng et al., 1996; Dragic et al., 1996). The precise mechanism of this interference is unknown.

The cellular factors that account for the inability of macrophage-tropic HIV-1 to enter EU cells have not been elucidated. Importantly, the $CD4^+$ T-cells of EU2 and EU3, while resistant to infection by macrophage-tropic virus, are readily infected by T-tropic HIV-1 (Paxton et al., 1996). Thus, the EU cells do not have a generalized inability to support virus replication. Presumably, they either lack a specific factor that is required for entry of macrophage-tropic HIV-1 or contain an inhibitor of this step of virus replication. T-cell clones derived from the PBMC of one of these individuals (EU2) generally secreted about 10-fold more β-chemokine than similar clones derived from control individuals (Dragic et al., 1996). Thus, the resistance of these cells to HIV-1 infection could be caused by autocrine or paracrine blocking of CKR-5 coreceptor activity by the high levels of endogenous chemokines. Alternatively, genetic alteration of CKR-5 itself could decrease its ability to mediate viral entry.

Here we investigate the genetic basis of the resistance of the cells from exposed–uninfected individuals EU2 and EU3 to HIV-1 infection. We report that both individuals have an identical homozygous defect in the gene encoding CKR-5. This defect, a 32 bp deletion in the region corresponding to the second extracellular loop of CKR-5, encodes a severely truncated molecule that fails to reach the cell surface. As a result, the cells are resistant to infection by macrophage-tropic virus. It is likely that the defect in CKR-5 is primarily responsible for the ability of these individuals to remain uninfected following repeated exposure to HIV-1. Furthermore these findings suggest an essential role for CKR-5 in the sexual transmission of HIV-1.

Results

Expression of Coreceptors in EU-Derived Cells

At least two mechanisms could account for the resistance of EU cells to infection by macrophage-tropic viruses. Overproduction of β-chemokines in these cells could lead to receptor desensitization and down-regulation of CKR-5, inhibiting its coreceptor function. Alternatively, a failure to synthesize functional cell surface coreceptor caused, for example, by inadequate transcriptional activity of the *CKR-5* gene or alteration of the *CKR-5* coding sequence could account for the resistance of these cells to infection. To assess the relative amounts of *CKR-5* and other coreceptor transcripts in EU cells, we used reverse transcription-polymerase chain reaction (RT–PCR) to amplify *CKR-5*, *fusin*, and *CKR-1* cDNAs from equivalent amounts of RNA isolated from four previously characterized EU or normal donor T-cell clones. EU2-derived cloned T-cell lines (EU2.11, EU2.15, EU2.16, EU2.17), and an EU3-derived T-cell clone (EU3.1) are resistant to macrophage-tropic virus, while two clones from unexposed control donors (LW4.39 and LW5.49) are sensitive to both macrophage-tropic and T-tropic virus (referred to as LW4.13 and LW5.8 in Dragic et al., 1996). Clones EU2.11, EU2.15, EU2.16, and EU3.1 were also found to be somewhat resistant to T-tropic virus, while clone EU2.17 was readily infectable (Dragic et al., 1996). Results of RT–PCR analysis showed that *CKR-5*, *CKR-1*, and *fusin* transcripts were at least as abundant in the EU2 and EU3 cells as in those of normal donors (Figure 1). *CKR-5* and *CKR-1* transcripts appeared to be present at slightly elevated levels in EU cells as compared with controls, perhaps due to decreased negative feedback control of transcription of these genes. Thus, the resistance of EU2 cells to macrophage-tropic HIV-1 was unlikely to be due to the absence or insufficient expression of the coreceptor gene.

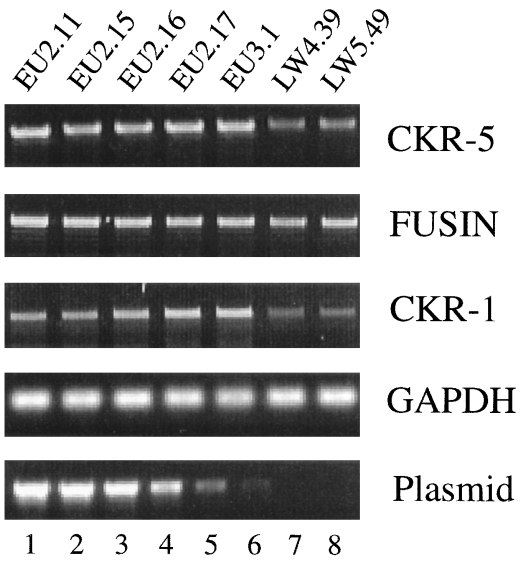

Figure 1. Expression of CKR-1, CKR-5, and Fusin mRNA in EU Cells

RT–PCR amplification of CKR-5, Fusin, CKR-1, glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts from the indicated EU or normal donor T-cell clones. All RNAs were treated with RNAase-free DNase before cDNA synthesis. To confirm the absence of contaminating DNA, control cDNA reactions were prepared in which reverse transcriptase was omitted from each reaction. Amplified products were not observed in these controls (data not shown). GAPDH served as control to confirm equal efficiency of amplification of each of the RNAs. Bottom panel shows PCR amplification of serial 10-fold dilutions of pcCKR5 plasmid DNA beginning with 1 ng. 10^0, (lane 1); 10^{-1} (lane 2); 10^{-2} (lane 3); 10^{-3} (lane 4); 10^{-4} (lane 5); 10^{-5} (lane 6); 10^{-6} (lane 7); 10^{-7} (lane 8).

CKR-5 Transcripts from EU Cells Do Not Encode Active Coreceptor

We previously showed that CKR-5 and Fusin coreceptor function can be sensitively detected in a transient transfection assay (Deng et al., 1996). In this assay, 293T human embryonic kidney cells are transfected with CD4

and CKR-5 expression vectors. The cells are then infected with single-cycle luciferase reporter virus derived from the HIV-1 provirus pNL-Luc-Env⁻ (Connor et al., 1995). This vector is a modified form of the HIV-1 provirus NL4-3 in which a firefly luciferase gene replaces *nef* and a frameshift mutation has been inserted in *env*. As a result of the frameshift in *env*, the virus is restricted to a single-cycle of replication. Following infection and integration of this virus, the luciferase reporter gene is expressed, reflecting the efficiency with which the virus entered. Pseudotyping this reporter virus with different HIV-1 Envs allows measurement of the relative activity of different coreceptors. Reporter viruses pseudotyped by macrophage-tropic Envs JRFL, BaL, or ADA are specific for CKR-5 while those pseudotyped by the T-tropic Env HXB2 use Fusin for entry. Reporter virus pseudotyped by amphotropic murine leukemia virus (A-MLV) Env (Page et al., 1990; Landau et al., 1991), which enters through a noncoreceptor pathway, was used in these experiments to rule out postentry effects on luciferase expression.

We used the transient transfection assay to test the coreceptor function of EU2 and EU3 CKR-5. *CKR-5* cDNAs were amplified from EU2, EU3, or normal donor RNA by RT–PCR and cloned into the expression vector pcDNAI/amp. Each CKR-5 expression vector was then mixed with an equal amount of CD4 expression vector and used to transfect 293T cells. Coreceptor function encoded by each CKR-5 expression vector was measured by infecting the transfected cells with the panel of single-cycle luciferase reporter viruses. The results showed that normal donor-derived CKR-5 expression vector encoded coreceptors that mediated efficient entry of macrophage-tropic virus (Figure 2A, WT). In contrast, the CKR-5 expression vectors derived from each of the five EU2 and EU3 cell lines were inactive. The failure of these expression vectors to encode functional CKR-5 is not likely to have been the result of misincorporation during RT–PCR. RT–PCR amplified *CKR-5* from control cells has in every case to date (at least four independent repetitions) resulted in functional *CKR-5* cDNA. In addition, RT–PCR amplification of *CKR-5* from EU2 or EU3 has not yielded a single functional cDNA in at least seven independent repetitions. Furthermore, *fusin* cDNA amplified from these same RNA preparations (described below) was uniformly active, arguing against a global defect in EU RNA. To determine whether the inactive *CKR-5* cDNAs encoded a product with dominant negative activity, equal amounts of active and inactive CKR-5 expression vector were mixed with CD4 expression vector and used to transfect 293T cells. The addition of inactive CKR-5 vector did not reduce the coreceptor activity of the wild-type CKR-5 (Figure 2A), arguing against a dominant negative role for the nonfunctional gene.

We used a similar approach to determine whether Fusin derived from EU cells was active as a coreceptor. *fusin* cDNAs were amplified by RT–PCR and cloned into the murine leukemia virus-based retroviral vector, pBABE-puro (Morgenstern and Land, 1990). These vectors were then used to establish HOS. CD4 (He and Landau, 1995) (human osteosarcoma cells expressing CD4) stably expressing EU2-derived Fusin. Coreceptor function of the expressed Fusins was then determined by infecting the cells with luciferase reporter viruses. The results showed that EU-derived *fusin* cDNAs encoded coreceptors that mediated entry of T-tropic HIV-1 at levels comparable to that of the wild-type (Figure 2B). Similar results were obtained with *fusin* cDNA derived from EU3 (data not shown). Thus, the cells of these

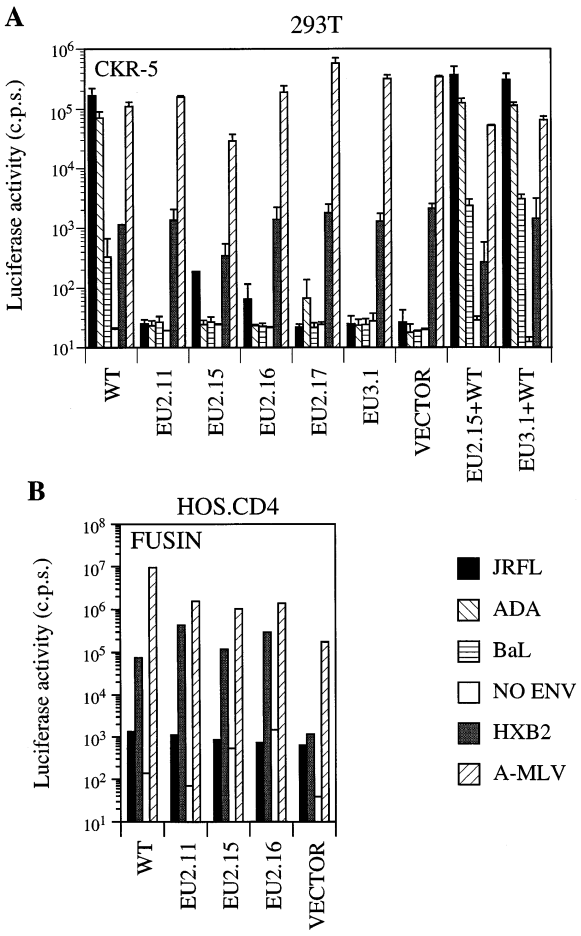

Figure 2. EU CKR-5 Does Not Function as an HIV-1 Coreceptor

(A) CKR-5 expression vectors were tested in transient assay for ability to mediate entry of macrophage-tropic virus. 293T cells were cotransfected with pcDNAI/amp expression vectors for CKR-5 cDNAs derived from indicated EU2, EU3, or normal donor cell lines and with CD4 expression vector pcCD4. To test for possible dominant negative activity, wild-type and EU-derived CKR-5 expression vectors were mixed in a 1:1 ratio and used to transfect 293T cells. The cells were infected with the indicated luciferase reporter viruses (10 ng $p24^{gag}$) pseudotyped by macrophage-tropic (JRFL, ADA, BaL), T-tropic (HXB2), or A-MLV Env. Luciferase activity was measured three days later as described in Experimental Procedures. This experiment has been repeated three times with similar results. Wild-type *CKR-5* has been amplified from three different cell lines and showed similar activity to that shown here. BaL Env typically results in significantly lower infectivity than the other two macrophage-tropic Envs (Deng et al., 1996). Error bars indicate standard deviation of duplicate independent measurements.

(B) HOS.CD4 cells stably expressing *fusin* cDNAs or containing control pBABE-puro vector alone were infected with luciferase reporter viruses as in (A). These cells have very low levels of endogenous Fusin expression and become very susceptible following transfection (data not shown).

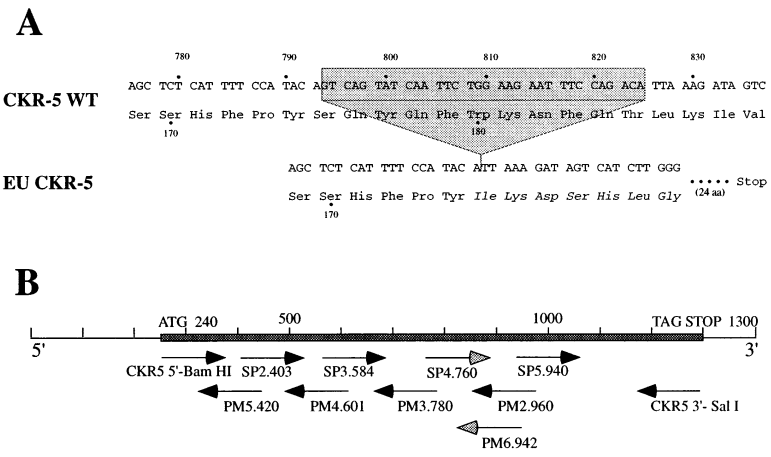

Figure 3. EU *CKR-5* Contains a 32 bp Internal Deletion

(A) The nucleotide sequences of wild-type *CKR-5* derived from normal donor (above) and EU cDNAs (below) are shown. The nucleotide sequence from normal donor *CKR-5* cDNA was determined for the complete 1055 bp coding sequence and found to be identical to that reported by Samson et al. (1996). The nucleotide sequence of 2 EU *CKR-5* cDNAs were identical to wild-type over the complete coding region with the exception of a 32 bp deletion. Nucleotide sequence of the 200 bp region of *CKR-5* encompassing the deletion was also determined for cDNAs derived from clones EU2.11, EU2.15, EU2.16, EU2.17, and EU3.1. Each contained the identical deletion with no additional nucleotide changes. Only the region flanking the deletion is shown. The deleted region (nt 794 to 825) is shaded. Amino acids encoded out of frame as a result of the deletion beginning at codon 185 are shown in italics. Nucleotide numbering (above) is from the first nucleotide of the reported *CKR-5* sequence (Samson et al., 1996). Amino acid residue numbers are shown below.

(B) Oligonucleotide primers used for nucleotide sequencing of *CKR-5* cDNA and detecting the deleted allele by PCR. The coding region is indicated by dark shading. Primers have been designated by nucleotide position at which they hybridize. Oligonucleotides used in PCR for detecting the deletion are shown as lightly shaded arrows. Oligonucleotides for cloning the full-length cDNA are shown at either end.

individuals are likely to contain fully functional Fusin. The reason that some T-cell clones from EU2 support T-tropic virus replication at levels somewhat reduced to that of control cells (Dragic et al., 1996) is not clear but could be due to a slight decrease in the amount of coreceptor on the cell surface or to increased synthesis of the yet uncharacterized Fusin ligand. In addition, *CKR-1* cDNA amplified from EU2 RNA was fully active in mobilizing intracellular calcium upon binding to RANTES or MIP-1α (data not shown). These findings strongly suggest that EU2 and EU3 express *CKR-5* mRNA that does not encode a functional HIV-1 coreceptor. Thus, the resistance of EU2 and EU3 to infection by macrophage-tropic HIV-1 strains is likely to be due to genetic alteration of *CKR-5*. Furthermore, the EU phenotype is not the result of a generalized defect in several coreceptors, but is likely to be restricted to a defect in *CKR-5*.

EU CKR-5 RNA Contains a 32 Base Coding Sequence Deletion

To define the predicted genetic alteration, we determined the nucleotide sequence of the complete 1055 bp coding region of *CKR-5* cDNA clones derived from EU2, EU3, and a normal donor. Nucleotide sequences were determined on both strands using the set of primers shown in Figure 3B. This analysis revealed an identical 32 bp deletion in each of the EU2 and EU3 cDNAs. The deletion spans nucleotides 794 to 825 in a region corresponding to the second extracellular loop of the receptor (Figure 3A). The deleted allele encodes a 215 amino acid protein (the wild-type receptor is 352 amino acids) in which the C-terminal 31 amino acids are translated out of frame. This deletion was present in all four independently amplified EU2 *CKR-5* cDNAs and in the two EU3 cDNAs sequenced. Each of six cDNAs amplified from normal control T cell clones LW4.39 and LW5.49 was identical to wild-type *CKR-5*, as was a single *CKR-5* amplified from PBMC of another control unexposed individual. In each EU-derived cDNA, no nucleotide changes were noted outside the 32 bp deletion, with a single exception. In one EU2-derived *CKR-5* cDNA, a single G to A change at nt 559 that encodes an Arg to Lys change was found. This alteration was not present in any of the three other EU2 *CKR-5* cDNAs sequenced and may have arisen by misincorporation during RT–PCR.

Defective CKR-5 Is Encoded in the Genomic DNA and Inherited

The 32 bp deletion found in EU *CKR-5* RNA could have resulted from an aberrant splice to cryptic splice sites or could have been due to the presence of the deletion in the *CKR-5* gene itself. To distinguish between these possibilities, we amplified a portion of *CKR-5* containing the deleted region from EU2, EU3, and control donor genomic PBMC DNA. PCR amplification using the flanking primers SP4.760 and PM6.942 (Figure 3B) yields predicted fragments of 182 bp and 150 bp for the wild-type and deleted alleles, respectively. PCR amplification from EU2 and EU3 genomic DNA with these primers showed only the 150 bp band (Figure 4, lanes 3 and 5). In contrast, only the 182 bp band was amplified from LW5 control donor DNA. These findings confirmed the presence of the *CKR-5* deletion in the genomic DNA of both EU individuals. In addition, we examined the *CKR-5* alleles of 13 of the remaining 23 EU individuals in the cohort. Only one other individual's $CD4^+$ cells were highly resistant to infection, and the genomic DNA of this individual had only deleted *CKR-5*. The other 22 samples were either fully or partially infectable (Paxton et al., 1996) and of those tested, only wild-type *CKR-5* allele was present (data not shown).

To rule out possible PCR-related artifacts, the *CKR-5* allele status of these individuals was confirmed by genomic Southern blot analysis. High molecular weight DNA isolated from PBMC of EU2, EU3, and EU3′s parent and a normal donor was cleaved with EcoRI and BglII restriction endonucleases. This releases a 283 bp wild-type or 251 bp deleted fragment. Southern analysis confirmed that EU2 and EU3 DNA contains only the deleted

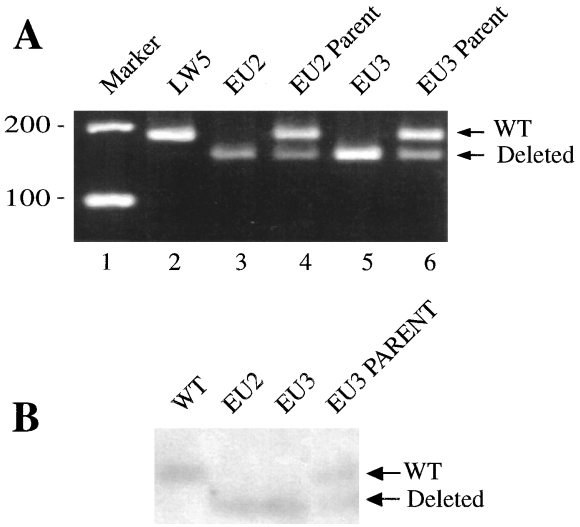

Figure 4. The EU *CKR-5* Deletion Is Present in Genomic DNA

(A) *CKR-5* was amplified by PCR from genomic DNA of indicated EU, EU parent, or normal control PBMC. Primers SP4.760 and PM6.942 (shown in Figure 3) that flank the deletion were used to generate wild-type and deleted fragments of 182 bp and 150 bp, respectively. DNA from the other parents was unavailable. Control reactions in which DNA was omitted from the PCR had no amplified product (data not shown). Sizes of marker fragments are indicated at left in base pairs.

(B) Genomic DNA (10 μg) isolated from PBMC of the indicated donors was digested with EcoRI and BglII and hybridized to a ^{32}P-labeled *CKR-5* probe. The position of the wild-type and deleted fragments is indicated by arrows.

allele, while the parent of EU3 has wild-type and deleted alleles (Figure 4B).

The finding that two unrelated EU individuals have identically deleted *CKR-5* alleles suggests that this mutation originated in a common ancestor and was inherited through the germline. Mendelian inheritance of these alleles would require that the parents of a homozygous EU individual are themselves either homozygous or heterozygous for the deletion. Alternatively, the deletion could have arisen *de novo* as the result of a recombination hot spot. To distinguish between these possibilities, we determined the *CKR-5* status of the available parental genomic DNA of these individuals. For both EU2 and EU3, the tested parent appeared to be heterozygous (Figure 4A, lanes 4 and 6), with one chromosome having the deleted and the other having the wild-type allele (DNA from the other parent of each individual was unavailable). Thus, it is likely that for both individuals each parent transmitted a deleted allele to their offspring. However, because of the unavailability of DNA from the other parent, we could not demonstrate this definitively.

To determine whether inheritance of the deleted *CKR-5* allele could have been sex-linked in EU2 and EU3, both of whom are males, we determined the chromosomal localization of the gene. *CKR-5* was amplified from genomic DNAs of two somatic cell hybrid mapping panels (data not shown). The first panel localized the *CKR-5* gene to chromosome 3. The second, a panel of radiation hybrids (Naylor et al., 1996), further localized the gene to the short arm of chromosome 3 in cytogenetic band 3p21. Thus, *CKR-5* is linked to other members of the chemokine receptor family including *CKR-1*, *CKR-2*, *V28*, and *CMKBRL1* that are also on 3p21 (Baggiolini et al., 1995; Combadiere et al., 1995; Raport et al., 1995). The autosomal localization of *CKR-5* predicts that its inheritance is not sex-linked.

EU2 and EU3 Are Homozygous for the *CKR-5* Deletion

The analysis described above is consistent with a homozygous *CKR-5* deletion in EU2 and EU3. However, it is equally consistent with a hemizygous status in which loss of a portion of one parental chromosome 3 resulted in complete loss of *CKR-5*. To distinguish between these possibilities, we analyzed the *CKR-5* locus in normal donor and EU2 genomic DNA. Genomic DNAs from the PBMC of a normal donor, from two normal donor T-cell clones (LW4.39 and LW5.49), and from an EU2 T-cell clone was cleaved with EcoRI, PvuII, or HindIII and hybridized to a full-length ^{32}P-labeled *CKR-5* probe. For all four genomic DNAs, the probe hybridized to bands of similar lengths with the following exceptions. First, EcoRI and HindIII digests of EU2 DNA showed *CKR-5* fragments of 1.2 kb and 2.0 kb, respectively, that were slightly smaller than those of the control DNAs (Figure 5, lanes 4 and 8, arrows). This decrease is consistent with the presence of the 32 bp deletion, further supporting the predicted presence of the deletion in the genomic DNA of EU2. Second, the *CKR-5* of LW5.49 contains a PvuII restriction fragment length polymorphism (RFLP) (Figure 5, lane 11). An additional PvuII site in this DNA results in the appearance of a 2.9 kb band in addition to the common 3.4 kb band. Both *CKR-5* bands are decreased in intensity as compared with that of the other three samples (Figure 5, compare lane 11 with lanes 9, 10, and 12). This decrease reflects the twofold decrease in copy number of each fragment in the heterozygote. Thus, the difference between a haploid or diploid *CKR-5* content is detectable in this analysis. Similarly, in the EcoRI and HindIII digests the *CKR-5* fragment of EU2 DNA is similar in intensity to that of the three control samples. In addition, Southern analysis of EU3 genomic DNA showed only the deleted *CKR-5* fragment (data not shown). Together, these findings suggested that EU2 and EU3 were homozygous for the deleted allele and that no gross rearrangement of chromosome 3 was associated with the deletion.

EU Cells Do Not Express Functional CKR-5

Serological reagents are not currently available to detect CKR-5. We therefore used two methods to investigate whether the EU cells expressed functional CKR-5. In the first, we tested whether epitope-tagged EU CKR-5 could be expressed on the cell surface. To do this we constructed vectors expressing EU2, EU3, or wild-type influenza hemagglutinin (HA)-tagged CKR-5. These were mixed with an equal amount of CD4 expression vector to control for transfection efficiency, and used to cotransfect 293T cells. Fluorescence-activated cell sorter (FACS) analysis of the cells stained with anti-HA MAb

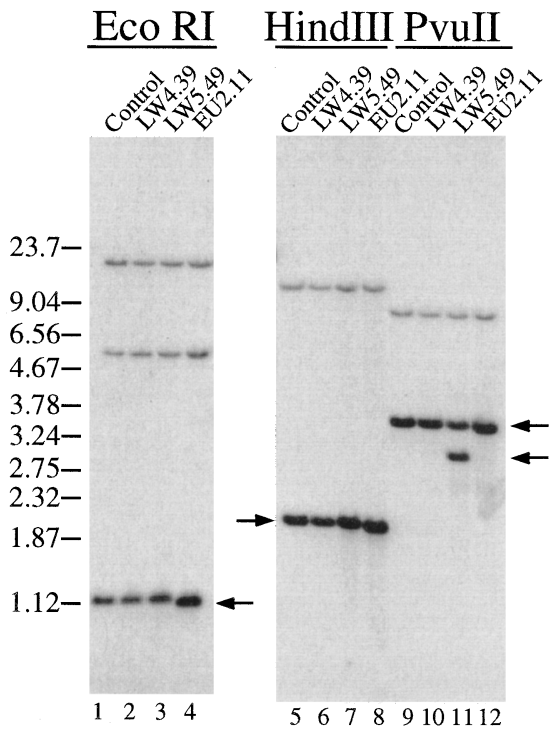

Figure 5. EU2 Is Homozygous for Deleted *CKR-5*

Genomic DNA from normal donor PBMC, from normal donor T-cell clones LW4.39 and LW5.49, and from EU2-derived T-cell clone EU2.11 was cleaved with indicated restriction enzyme. The cleaved DNA was separated by agarose gel electrophoresis and hybridized to a ^{32}P-labeled *CKR-5* cDNA probe. Arrows indicate *CKR-5* fragments. The 22 kb band in the EcoRI digests is derived from a fragment containing the 3′-end of *CKR-5*. The other faint high molecular weight bands are likely to correspond to *CKR-2*, which is closely related in nucleotide sequence. High stringency washing of similar filters in pilot experiments removed these bands. *CKR-5* bands from EU2 are equal in intensity to those of controls, suggesting diploid content. Intensity of a fragment with a haploid content is shown by PvuII digest of LW5.49 who is heterozygous for a PvuII RFLP. Molecular size markers are indicated at left in kilobases.

revealed that HA-tagged wild-type CKR-5 was expressed at the cell surface (Figure 6A, upper right). Infection studies showed that this protein retained its coreceptor activity (data not shown). In contrast, HA-tagged CKR-5 derived from EU2 or EU3 was not detected (Figure 6A, lower panels). Immunoblot analysis of the transfected cells suggested that the mutant protein was not stably expressed in the cytoplasm (Figure 6B).

EU2 Cells Do Not Transduce CKR-5-Mediated Signals

To determine whether PBMC from EU individuals express functional chemokine receptors, we measured intracellular [Ca^{2+}] levels in response to challenge with the β-chemokines MIP-1β and RANTES. When control cells were loaded with the calcium probe Fura 2 and challenged with physiological concentrations of MIP-1β followed by RANTES, a rapid increase in intracellular [Ca^{2+}] was observed (Figure 6C). In contrast, PBMC from EU2 were refractory to MIP-1β but responded to RANTES (Figure 6C). Thus, EU2 cells do not transduce CKR-5-mediated signals, yet are fully competent to transduce signals through other chemokine receptors. EU2 cells therefore do not have a generalized deficiency in their ability to transduce signals from other chemokine receptors.

Deleted *CKR-5* Heterozygotes Are Common and Their Cells Can Be Infected In Vitro

Resistance to HIV-1 infection is rare. Of a large number of PBMCs from random blood donors, all were readily infected by T-tropic and macrophage-tropic HIV-1 (Spira and Ho, 1995, and data not shown). Because the pool size from which EU2 and EU3 were selected is unknown, we were not able to estimate the frequency of resistant individuals. To estimate the allele frequency in a population with a genetic background similar to that of EU2 and EU3 (both are of European descent), we tested a panel of genomic DNAs (MacDonald et al., 1991, 1992) isolated from unrelated individuals of western European origin. Of the 44 samples tested, 10 (22.7%) were heterozygous for the deletion, and the rest showed only the wild-type allele (Figure 7A). The heterozygous status of these samples was confirmed by genomic Southern analysis (Figure 7B and data not shown). Two other panels of genomic DNAs (MacDonald et al., 1991, 1992) isolated from individuals of western European origin showed a 15.6% (5 out of 32) and 19.6% (9 out of 46) frequency of heterozygotes (data not shown). Thus, among the 122 samples tested, 24 were heterozygous yielding a calculated allele frequency of 0.098. Assuming Hardy-Weinberg equilibrium, this predicts frequencies of 81% wild-type homozygotes, 18% heterozygotes, and 1% homozygotes for deleted *CKR-5* in populations of western European heritage. In a preliminary analysis of individuals from other backgrounds, genomic DNAs from members of the Venezuela reference mapping resource (Locke et al., 1988; Tanzi et al., 1988) were tested. No deleted alleles were present in the 46 genomic DNAs tested. Thus, the allele is common in some human populations but much rarer in others. These findings suggest a rather recent evolutionary origin of this mutation.

Individuals that are heterozygous for the *CKR-5* deletion could express less coreceptor and as a result replicate virus less efficiently. In a preliminary analysis of the infectability of heterozygous cells, we measured the replication efficiency of a macrophage-tropic HIV-1 strain, SF162, on the $CD4^+$ T-cells of EU2, EU3, and one parent of each. The cells of LW5 were used as a source of wild-type cells. In this analysis, virus replicated efficiently on the wild-type cells, failed to replicate on the EU cells, and replicated to an intermediate level on both parental T-cells (Figure 7C). This finding suggested that heterozygous cells are somewhat reduced in their ability to replicate macrophage-tropic HIV-1.

Discussion

We show here that two individuals who are resistant to HIV-1 infection in spite of repeated exposures are homozygous for a defect in the gene encoding CKR-5, a major coreceptor for macrophage-tropic HIV-1 isolates.

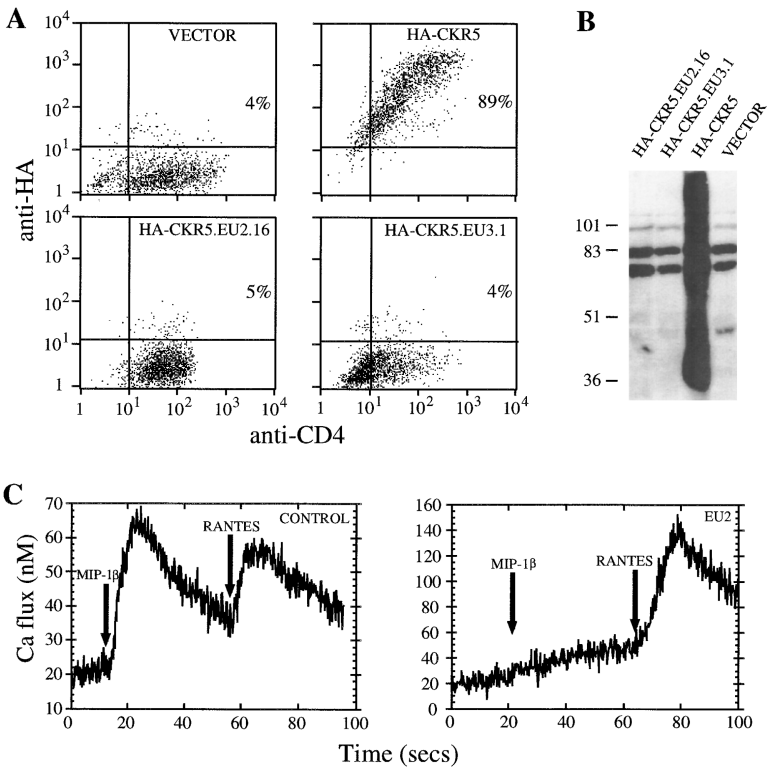

Figure 6. EU CKR-5 Is Not Detectable at the Cell Surface or in the Cytoplasm and Does Not Transduce Intracellular Signals

(A) 293T cells were cotransfected with equal amounts of CD4 expression vector pcCD4 and indicated wild-type CKR-5 (HA-CKR5), EU CKR-5 (HA-CKR5.EU2.16 and HA-CKR5.EU3.1), or control pcDNAI/amp vector. Two days later, the cells were stained with FITC-conjugated anti-CD4 MAb Leu3a, anti-HA MAb 12CA5, and phycoerythrin-conjugated second antibody. Expression vectors for EU HA-CKR-5 were derived from T-cell clones EU2.16 and EU3.1, as indicated. The number of cells staining positive for both the HA epitope and CD4 is indicated as the percentage of cells falling in the upper right quadrant. Untransfected cells did not stain significantly with either antibody (data not shown).

(B) The transfected cells were lysed and cytoplasmic HA-tagged CKR-5 was detected on immunoblots probed with anti-HA MAb. CKR-5 appears as an extremely heterogeneous band extending from 40 kDa to the origin. The heterogeneous mobility of CKR-5 on SDS–PAGE appears to be a property of this family of proteins since similar results were found on immunoblots probed for CKR-1 (data not shown).

(C) EU cells fail to increase intracellular [Ca^{2+}] in response to MIP-1β. PBMC from EU2 or normal control donor were loaded with the calcium probe Fura 2 and exposed to MIP-1β and then RANTES (100 nM each) at the times indicated by arrows. Intracellular [Ca^{2+}] was measured by spectrofluoremetry as described in Experimental Procedures. Chemokine binding assay showed that cells from EU2 bound 2- to 3-fold less MIP-1β than control. Both bound similar amounts of RANTES (data not shown).

Both individuals have identical *CKR-5* alleles containing a 32 bp deletion in a region of the gene corresponding to the second extracellular loop. The deletion results in a frameshift that encodes a severely truncated protein which is not detected at the cell surface or in the cytoplasm. The absence of CKR-5 from these cells explains their inability to transduce signals in response to MIP-1β. The reason for their enhanced secretion of β-chemokines is not clear, but could have to do with decreased up-take of chemokines as a results of the missing chemokine receptor or with increased chemokine expression due to a lack of negative regulatory signals that normally reduce chemokine synthesis.

These findings suggest that the homozygous deletion in *CKR-5* accounts for the resistance of these two individuals to transmission of HIV-1. While it is conceivable that a second, yet undetected defect contributed to their resistance, the finding that the EU cells were fully infectable by A-MLV pseudotypes and by T-tropic HIV-1 argues against the presence of a secondary defect. The *CKR-5* deletion is not, however, likely to account for the resistance to infection of all EU individuals. Individuals whose cells are only partially resistant to primary HIV-1 infection could express CKR-5 alleles with reduced coreceptor activity. EU individuals whose cells were fully infectable may resist infection by a yet undefined mechanism.

Both individuals are healthy, without any obvious clinical conditions. The absence of a phenotype associated with the *CKR-5* defect may result from the redundant nature of the chemokine system. Several members of the chemokine receptor family have overlapping ligand reactivities and tissue distribution. Thus, in individuals homozygous for the defective allele, the loss of CKR-5 might be compensated by a chemokine receptor such as CKR-1, that has a similar ligand profile. By analogy, rare individuals have been reported that do not express the erythrocyte chemokine receptor, DARC (Duffy antigen), as a result of a 14 bp deletion in the coding sequence (Mallinson et al., 1995). As in the case of CKR-5, no phenotype was associated with that deletion.

Frequency and Evolution of the Deleted *CKR-5* Allele

The deleted allele was present in a surprisingly high percentage of unrelated individuals of western European heritage (~20% heterozygotes). In contrast, it was present at much lower frequency in a panel of individuals from Venezuela. Assuming Hardy-Weinberg equilibrium, the frequency of *CKR-5*-deleted homozygotes is about 1% in the general population of persons with western European heritage. The *CKR-5* allele status of HIV-1 infected individuals remains to be determined. Presumably, infected individuals who are homozygous for deleted *CKR-5* are extremely rare. If transmission through a CKR-5-independent route occurs infrequently, homozygous individuals would not be completely protected. A large-scale analysis to determine the frequency of the deleted CKR-5 allele among HIV-1 infected individuals

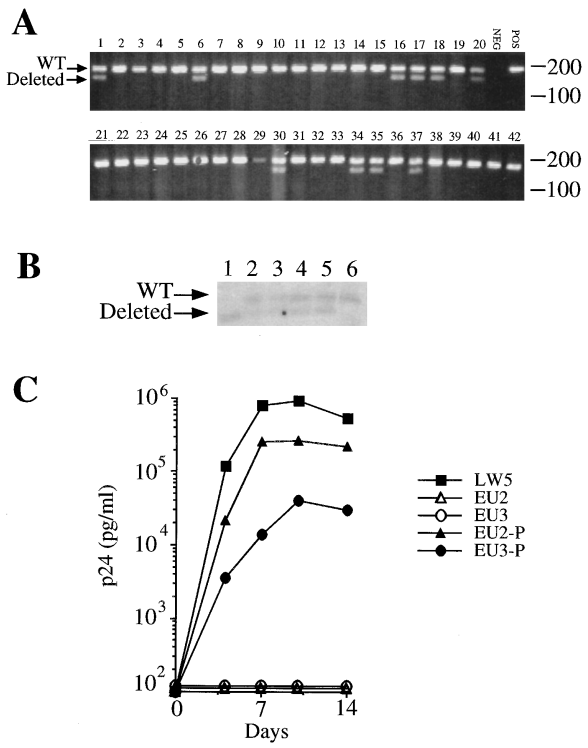

Figure 7. The Deleted *CKR-5* Allele Is Frequent in Some Populations

(A) *CKR-5* alleles amplified from 42 genomic DNAs from individuals of western European heritage in the CEPH reference mapping resource (MacDonald et al., 1991) (excluding codes 102 and 104). *CKR-5* sequences were amplified using primers SP4.760 and PM6.942 (shown in Figure 3) flanking the 32 bp deletion, generating wild-type and deleted fragments of 182 bp and 150 bp, respectively. Positions of markers are indicated at right in base pairs. PCR products were separated on 4% Metaphor agarose. Reactions containing no genomic DNA showed no amplified product (NEG). PCR amplification of pcCKR5 plasmid DNA showed only the 182 bp band (POS). Weak amplification of sample 29 may have been due to low genomic DNA concentration. In heterozygous samples (1, 6, 16, 17, 18, 20, 30, 34, 35, 37) the bands have decreased intensity as compared with homozygotes, as expected. The deleted allele is always less intense than the wild-type band, possibly as a result of its smaller size. Two samples that amplified weakly but were wild-type are not shown. Thus, the frequency of heterozygous individuals in this population is 22.7%.

(B) Southern analysis of selected heterozygous samples. Genomic DNA from T-cell clone EU2.15 (lane 1), selected heterozygous samples 30 (lane 2), 34 (lane 3), 35 (lane 4), and 37 (lane 5) and a control donor were cleaved with BglII and EcoRI and hybridized to a full-length [^{32}P-α]dCTP-labeled *CKR-5* probe. Wild-type and deleted *CKR-5* fragments are indicated.

(C) Growth of macrophage-tropic HIV-1 on CD4$^+$ cells heterozygous for *CKR-5*. Activated CD4$^+$ cells from indicated donors were infected with macrophage-tropic HIV-1 strain SF162. Virus replication was measured by determining the amount of p24^{gag} present in the cultures at the indicated days postinfection. This experiment has been repeated with similar results using luciferase reporter viruses.

would permit calculation of the extent to which homozygosity protects against infection.

It is likely that the deleted *CKR-5* allele in the majority of heterozygous and homozygous individuals is inherited. This was suggested by our finding that EU2 and EU3 both had at least one heterozygous parent. Furthermore, our finding that the deletion was identical in every individual makes it unlikely that a recombination hot spot continually generates these alleles in the population. It is difficult to estimate when this deletion first occurred; however, its restricted distribution and the absence of secondary mutations in the gene suggest a rather recent evolutionary origin.

There is no reason to believe that the high frequency of heterozygotes among individuals of western European ancestry was due to selection pressure from the virus since HIV-1 has only recently become endemic to this population. However, it is possible that in areas where HIV-1 has been endemic for considerably longer periods of time, a selective advantage would be provided to individuals heterozygous or homozygous for this or similar *CKR-5* alleles that may be present in these populations. Precedence for selective pressure to lose expression of a chemokine receptor is provided by the example of the erythrocyte chemokine receptor, DARC. A mutant DARC allele that cannot be expressed in erythrocytes provides resistance to Plasmodium vivax (Horuk et al., 1993). Individuals carrying this allele may have a selective advantage in areas where the parasite is endemic.

Whether there is an advantage to heterozygosity for deleted *CKR-5* is not clear. While heterozygous cells had a somewhat reduced ability to replicate HIV-1 (4- to 10-fold less virus production), it is not clear whether this magnitude of decrease would offer significant protection against sexual transmission. Whether there is any protection offered to heterozygotes could be determined by comparing the frequency of the deleted allele in HIV-1-infected, HIV-1-exposed but uninfected, and nonexposed individuals. Such an advantage has been described in the case of β-globin mutations. The erythrocytes of individuals heterozygous for a mutation in β-globin are resistant to Plasmodium falciparum. As a result, the frequency of the mutant allele is high in sub-Saharan Africa (Wiesenfeld, 1968). Selection for the deleted *CKR-5* allele, or for other yet unknown *CKR-5* polymorphisms might be most evident in areas of Africa that have a high incidence of HIV-1 infection.

Implications for Pathogenesis and Transmission of HIV-1

In vitro, seven transmembrane domain proteins in addition to CKR-5, such as Fusin, CKR-2B, and CKR-3, have been shown to act as coreceptors for various HIV-1 isolates. The relative frequency with which each of these coreceptors is used in vivo is not known. Our findings on the resistance of multiply exposed individuals to HIV-1 infection demonstrate the critical role of CKR-5 in vivo. It is highly likely that HIV-1 transmission typically proceeds through a CKR-5-dependent event. Fusin and at least some of the other CKR genes appeared to be unaltered in the EU individuals. Thus, these receptors appear to be insufficient for sexual transmission of common strains of the virus.

Given their high numbers of exposures, it is likely that the EU individuals have been exposed to NSI, as well as SI viruses. These individuals have functional Fusin and other HIV-1 coreceptors, yet do not become infected. It may be that CD4$^+$ cells expressing the other coreceptors are not prevalent among the cells that line the mucosal surfaces at which transmission occurs. Alternatively, transmission followed by systemic spread

of virus may be supported only by specific cell-types that express CKR-5 but not Fusin or other potential HIV-1 coreceptors. Infection by SI virus of $CD4^+$ T-cells could occur transiently following sexual contact but may not result in systemic spread of the virus. Further replication of the virus might require infection of specialized cells such as macrophages that cannot be infected by SI viruses which use Fusin as coreceptor. The properties of CKR-5^+ cells such as macrophages that allow them to support systemic spread of HIV-1 are not yet defined, but may be related to their ability to activate T-cells. Transmission of SI virus could result in an abortive infection that is sufficient to establish anti-viral cytolytic T-cells, but which is soon cleared. Rowland-Jones et al. (1995) have recently described a cohort of Gambian prostitutes that have been heavily exposed to HIV-1 but remain uninfected. This phenomenon was associated with a cellular immune response against the virus. The presence of cytotoxic T lymphocyte responses against HIV-1 in these individuals is consistent with a transient infection. It will be important to determine the *CKR-5* status of such individuals.

Conclusions and Perspectives

This study highlights the critical importance of CKR-5 for HIV-1 transmission. CKR-5 is likely, in addition, to play an important role during the early phases of infection and possibly throughout the course of the disease. *CKR-5* could also play a role in determining the rate of disease progression. Some HIV-1-infected individuals remain asymptomatic, with very low viral burdens for unusually long periods of time (Cao et al., 1995; Pantaleo et al., 1995). It will be important to determine whether such individuals tend to be heterozygous for deleted *CKR-5*. Decreased levels of functional CKR-5 could be responsible for the low viral burdens that have been reported in these individuals. Alternatively, these individuals could have *CKR-5* alleles that encode receptors with reduced ability to mediate HIV-1 entry. Such alleles have not yet been detected but their existence is suggested by previous findings that the PBMC of some EU individuals have a moderate decrease in their ability to support macrophage-tropic HIV-1 replication (Paxton et al., 1996).

The presence of a nonfunctional *CKR-5* allele in individuals who are resistant to HIV-1 infection provides insight into the mechanisms governing virus transmission and may in the future provide a more complete understanding of the factors controlling disease progression. The lack of any obvious clinical conditions associated with the absence of functional CKR-5 suggests that this receptor is dispensable. These findings highlight the importance of developing therapeutic agents directed against the HIV-1–CKR-5 interaction. In addition, following the changing frequencies of *CKR-5* alleles in heavily infected populations may provide a unique insight into the complex genetic interplay between this pathogen and its host.

Experimental Procedures

RT–PCR and DNA Sequencing

Total cellular RNA was prepared using Triazol (GIBCO–BRL) according to the manufacturer's instructions and treated with 10 U RNAase-free DNAase (Boehringer-Mannheim). Oligo-dT-primed cDNA was prepared from 5 μg RNA using Superscript reverse transcriptase (GIBCO–BRL) according to the manufacturer's instructions and resuspended in 80 μl TE (10 mM Tris [pH 8.0], 1 mM EDTA). Aliquots of the cDNA (5.0 μl) were amplified with Taq polymerase (Boehringer-Mannheim) by 5 cycles of PCR (94°C, 30 s; 55°C, 45 s; 72°C, 90 s) followed by an additional 35 cycles (94°C, 30 s; 62°C, 45 s; 72°C, 90 s) in a volume of 50 μl using primers hybridizing to the 5′ and 3′ untranslated regions of *CKR-5*. The reaction products (10 μl) were separated on 1% agarose (FMC) in the presence of 0.5 μg/ml ethidium bromide and photographed. A control amplification containing no added DNA was included in each experiment reaction. No PCR product was detected in these reactions. For cloning of amplified cDNAs, PCR was carried out similarly except that instead of Taq polymerase a high fidelity Expand polymerase (Boehringer-Mannheim) was used for a total of 37 cycles. Amplified products were digested with BamHI and SalI restriction enzymes for *CKR-5* or HindIII and XhoI for Fusin. *CKR-5* cDNAs were cloned into the cytomegalovirus promoter-driven expression vector pcDNAI/amp (Invitrogen Corporation). *fusin* cDNAs were cloned into the retroviral expression vector pBABE-puro (Morgenstern and Land, 1990). Nucleotide sequencing of the cloned cDNAs was determined by the dideoxy method on both strands using primers shown in Figure 3B. Upstream and downstream oligonucleotide primers for amplifying and cloning were as follows: CKR-5: 5′-CTCGGATCCGGTGGA ACAAGATGGATTAT, 5′-CTCGTCGACATGTGCACAACTCTGACTG; Fusin, 5′-GGCTAAAGCTTGGCCTGAGTGCTCCAGTAGCC; 5′-CGT CCTCGAGCATCTGTGTTAGCTGGAGTG; CKR-1, 5′-GCGGATCCC AAAGTCCCTTGGAACCAGAG, 5′-GGTCTAGACAGGCCACCATTAC ATTCCCT.

Coreceptor Activity Analysis

Transient assay for CKR-5 coreceptor activity has been previously described (Deng et al., 1996). In brief, 293T cells were transfected by $CaPO_4$ coprecipitation with a mixture of pcCKR5 and pcCD4 (10 μg each) as described. The next day the cells were transferred to 24-well tissue culture dishes (1×10^4 per well), and the following day the cells were infected with luciferase reporter viruses (10 ng $p24^{gag}$). Reporter viruses pseudotyped by macrophage-tropic, T-tropic, or amphotropic Envs were prepared by transfecting 293T cells with NL-Luc-Env^- and the appropriate Env expression vector (10 μg each) and quantitated as previously described and frozen in aliquots at −80°C. Lysates (100 μl) were prepared two days later and luciferase activity in 20 μl was measured with commercial reagents (Promega) with a Packard Topcount scintillation counter. Fusin coreceptor activity was measured in a stable assay as described previously. In brief, retrovirus stocks were prepared from the pBABE-puro Fusin plasmids using the method of Landau and Littman (1992). HOS-T4 (human osteosarcoma cells expressing human CD4) were infected with the pBABE-puro viruses (2 ml supernatant). Two days later the cells were selected in 1.0 μg/ml puromycin. When the cells became confluent (about 5–7 days later), the cells were infected with luciferase reporter viruses and luciferase activity was measured as described above for the transient assay.

Southern and PCR Analysis of Genomic DNA

Genomic DNA was purified from cell lines and PBMC by standard methods. DNA (10 μg) was cleaved with restriction enzyme and separated by electrophoresis on 2% agarose gels. The DNA was transferred to nitrocellulose filters and hybridized to a BamHI–SalI cleaved *CKR-5* cDNA insert labeled with [α-^{32}P]dCTP by the random primer method using commercial reagents (Boehringer-Mannheim). PCR of genomic DNA was as described for cDNA except that 0.5–1 μg of genomic DNA was used instead of reverse transcriptase product. Amplified products were separated on 4% MetaPhor agarose (FMC) and visualized by ethidium bromide staining.

FACS and Immunoblot Analysis

A CKR-5 expression vector containing an HA epitope-tag (Field et al., 1988) near the amino terminus was constructed using wild-type or EU cell-derived CKR-5. These plasmids were mixed with an equal amount (10 μg each) of pcCD4 (Lenburg and Landau, 1993) and then used to transfect 293T cells. After two days, the 0.5×10^6 cells were stained with 0.5 μg monoclonal antibody (MAb) 12CA5

(BabCo), 0.2 μg phycoerythrin-conjugated rabbit anti-mouse immunoglobulin (Boehringer-Mannheim) and 0.5 μg FITC-conjugated anti-CD4 MAb Leu3a. Construction and characterization of the epitope-tagged wild-type CKR-5 will be reported elsewhere. The cells (10,000) were analyzed on a Becton-Dickenson FACScaliber. Neither antibody showed fluorescence levels above background on untransfected control 293T cells (data not shown). Tagged CKR-5 was detected on imunoblots as described previously (Paxton et al., 1993)

Chemokine-Induced Signal Transduction Measurement

PBMC were incubated for 4 min in phosphate-buffered saline (pH 3.0) to remove endogenously bound chemokines. In pilot experiments this removed 94% of the bound chemokine. The cells were centrifuged at 500 × G for 5 min and resuspended in Hanks' buffer for intracellular [Ca^{2+}] measurements. Intracellular [Ca^{2+}] was measured by spectrofluoremetry with Fura 2 as described (Neote et al., 1993), with the following modifications. Fura 2 was used at a 5 μM and 3 × 10^6 cells were used per assay.

HIV-1 Growth Curves

Replication of HIV-1 was measured as described previously (Paxton et al., 1996). In brief, PBMC (2 × 10^5) were from Ficoll purified leukocytes. The $CD4^+$ cells were purified and stimulated with phytohemagglutinin for three days. HIV-1 SF162 (600 TCID) was added for at least 4 hr, and the cells were washed three times to remove input virus. Samples were removed for $p24^{gag}$ quantitation by commercial ELISA (Abbot) at indicated days postinfection.

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