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Changes in the V3 region of gp120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Δ32 heterozygote

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Abstract

Heterozygosity for the CCR5 Δ32 allele is associated with delayed progression to AIDS in human immunodeficiency virus type 1 (HIV-1) infection. Here we describe an unusual HIV-1 isolate from the blood of an asymptomatic individual who was heterozygous for the CCR5 Δ32 allele and had reduced levels of CCR5 expression. The primary virus used CCR5, CXCR4, and an unusually broad range of alternative coreceptors to enter transfected cells. However, only CXCR4 and CCR5 were used to enter primary T cells and monocyte-derived macrophages, respectively. Full-length Env clones had an unusually long V1/V2 region and rare amino acid variants in the V3 and C4 regions. Mutagenesis studies and structural models suggested that Y308, D321, and to a lesser extent K442 and E444, contribute to the broad coreceptor usage of these Envs, whereas I317 is likely to be a compensatory change. Furthermore, database analysis suggests that covariation can occur at positions 308/317 and 308/321 *in vivo*. Y308 and D321 reduced dependence on the extracellular loop 2 (ECL2) region of CCR5, while these residues along with Y330, K442, and E444 enhanced dependence on the CCR5 N-terminus compared to clade B consensus residues at these positions. These results suggest that expanded coreceptor usage of HIV-1 can occur in some individuals without rapid progression to AIDS as a consequence of changes in the V3 region that reduce dependence on the ECL2 region of CCR5 by enhancing interactions with conserved structural elements in G-protein-coupled receptors.

Keywords: HIV-1; CCR5 Δ32; Env; V3; CCR5

Introduction

Human immunodeficiency virus type 1 (HIV-1) enters cells via interaction of the viral envelope glycoproteins (Env) with CD4 and a coreceptor. Macrophage (M)-tropic HIV-1 viruses primarily use CCR5 (R5) as a coreceptor, whereas T cell line-

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tropic HIV-1 viruses use CXCR4 (X4) (Berger et al., 1999; Doms and Trono, 2000; Moore et al., 2004). Dual-tropic viruses (R5X4) use both coreceptors. A subset of viruses can also use alternative coreceptors including CCR3, CCR2b, CCR8, Apj, Strl33 (BONZO/CXCR6), Gpr1, Gpr15 (BOB), CX3CR1, ChemR23, or RDC1 for entry (Berger et al., 1999; Doms and Trono, 2000; Moore et al., 2004). However, usage of coreceptors other than CCR5 and CXCR4 by primary viruses *in vitro* is rare (Zhang et al., 1998), and infection of primary cells occurs, with few exceptions, exclusively via CCR5 or CXCR4 (Cilliers et al., 2005; Moore et al., 2004). R5 strains predominate during primary infection and the asymptomatic phase, whereas expansion of viral coreceptor usage and emergence of X4 or R5X4 strains is frequently associated with rapid disease progression.

Delayed or slow HIV-1 disease progression can be defined by the lack of development of an AIDS defining illness for at least 10 years after infection with a slowly declining CD4+ T cell count. Viral genetic factors associated with slow progression or nonprogression include mutations in the HIV-1 *gag*, *rev*, *vif*, *vpr*, *vpu*, *env* and *nef* genes (Churchill et al., 2004, 2006; Deacon et al., 1995; Kirchhoff et al., 1995; Michael et al., 1997; Shioda et al., 1997; Wang et al., 2000). Host genetic factors linked to a delay in the onset of AIDS and prolonged survival include the CCR5 Δ32 mutation, CCR2b-V64I polymorphism, and certain HLA haplotypes (Dean et al., 1996; Eugen-Olsen et al., 1997; Huang et al., 1996; Smith et al., 1997) (reviewed in O'Brien and Moore, 2000; Roger, 1998). The CCR5 Δ32 mutation, which results in a 32-nucleotide deletion, is common in Caucasians, with heterozygosity in 15 to 20% and homozygosity in 1%. Individuals homozygous for the CCR5 Δ32 allele are highly resistant to HIV-1 transmission (O'Brien and Moore, 2000), whereas heterozygotes are susceptible but typically have delayed CD4+ T cell decline and prolonged survival compared to CCR5 wt/wt individuals (Dean et al., 1996; Eugen-Olsen et al., 1997; Huang et al., 1996; Michael et al., 1997). Among CCR5 Δ32/wt heterozygotes, there is large variation in levels of CCR5 expression (Cohen et al., 1997; de Roda Husman et al., 1999). Slow progression of HIV-1 disease has been correlated with reduced levels of CCR5 expression on CD4+ T lymphocytes and monocytes compared to levels in CCR5 wt/wt individuals (Cohen et al., 1997; de Roda Husman et al., 1999). Nonetheless, there is considerable overlap between CCR5 expression levels in CCR5 Δ32/wt heterozygotes and individuals with the CCR5 wt/wt genotype (de Roda Husman et al., 1999).

In this study, we isolated and characterized HIV-1 from the blood of an asymptomatic individual who was heterozygous for the CCR5 Δ32 allele and had reduced levels of CCR5 cell surface expression. In addition to using CCR5 and CXCR4, the virus has highly expanded utilization of alternative coreceptors that is broader than that of any previously described HIV-1 virus. Mutagenesis studies and structural models suggested Y308 and D321 in the V3 region of gp120, and to a lesser extent K442 and E444 in the C4 region, contribute to the broad coreceptor usage of Envs cloned from the viral isolate. Furthermore, studies using mutant CCR5 coreceptors indicated that Y308, D321, Y330, K442, and E444 alter dependence on the N-terminal and extracellular loop 2 (ECL2) regions of CCR5. The results suggest that expanded coreceptor usage of HIV-1 can occur in some individuals without rapid progression to AIDS as a consequence of changes in the V3 region that enhance interactions with conserved structural elements in G-protein-coupled receptors (GPCRs).

Results

Clinical history and isolation of HIV-1

The subject is a homosexual male who was infected with HIV-1 via sexual contact and first tested seropositive for HIV-1 in May 1989. As of 2006, the subject remained asymptomatic with no AIDS defining illness. His antiretroviral therapy (ART), plasma HIV-1 RNA levels, and CD4 counts are summarized in Supplementary Table 3. The subject was seropositive for cytomegalovirus, hepatitis A, hepatitis C, and *Toxoplasma gondii*. Genetic analysis of CCR5 alleles by PCR demonstrated heterozygosity for the CCR5 Δ32 deletion (data not shown). Two-color FACS staining of peripheral blood mononuclear cells (PBMC) collected in October 2003 demonstrated that the mean percentage of CCR5+ cells in the CD4+ T lymphocyte fraction was 0.9% ($n=2$, SD=0.08) as compared with 19.3% in healthy HIV-1-negative control subjects ($n=7$, SD=10.15). HIV-1 was isolated from PBMC collected in August 2000 by coculture with CD8-depleted donor PBMC as described (Gorry et al., 2001). Attempts to isolate HIV-1 from cryopreserved PBMC collected in October 1998 and March 1999 were unsuccessful.

Coreceptor usage

The ability of the primary virus isolate to utilize CCR5, CXCR4, or alternative coreceptors for virus entry was first determined in canine Cf2-Luc cells (Gorry et al., 2001) (Fig. 1A). The X4 NL4-3, R5 ADA, and R5X4 89.6 HIV-1 viruses were used as positive controls (Gorry et al., 2001, 2002b). The primary virus used both CCR5 and CXCR4 for virus entry. Efficient usage of CCR2b, CCR3, CCR8, Gpr1, Gpr15, Strl33 and Apj was also demonstrated. Compared to 89.6, usage of CCR2b, Gpr1, and Apj by the primary virus was approximately 5-, 20- and 2-fold greater, respectively. Compared to ADA, usage of CCR8, Gpr15 and Strl33 by the primary virus was approximately 5-, 6- and 2-fold greater. The virus could also enter canine Cf2-Luc cells transfected with CD4 alone, albeit at low levels. Coreceptor usage in human U87 astrocytoma cells was similar to that in Cf2-Luc cells with the exception that virus entry was not detected in U87 cells transfected with CD4 alone (data not shown), suggesting that entry in Cf2-Luc cells expressing CD4 alone was mediated by an endogenous canine coreceptor. The promiscuous pattern of coreceptor usage resembles that of some SIV and HIV-2 virus strains (Edinger et al., 1998; Morner et al., 1999; Reeves et al., 1997, 1999; Rucker et al., 1997), which can infect certain cell types lacking CD4. However, virus entry mediated by CCR3, CCR5, CXCR4, or the endogenous coreceptor expressed on Cf2-Luc

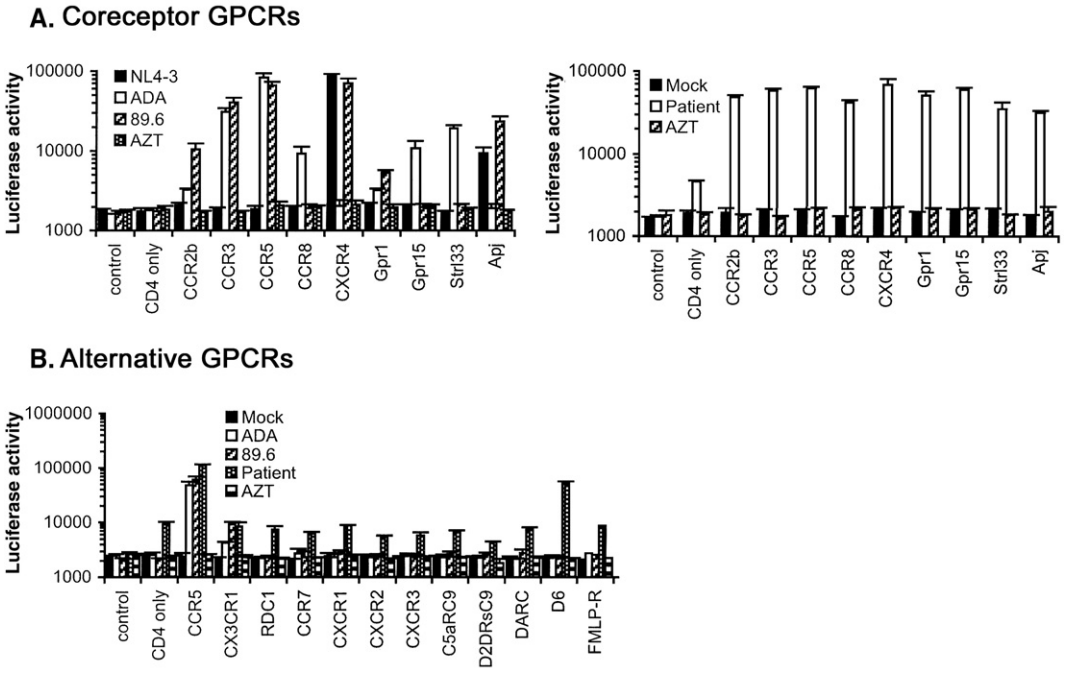

Fig. 1. Coreceptor usage. (A) Cf2-Luc cells were transfected with pcDNA3-CD4 alone or cotransfected with pcDNA3-CD4 and pcDNA3 expressing CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33 or Apj and infected with equivalent amounts of each control HIV-1 virus (left panel) or patient-derived virus (right panel). Control cells were transfected with pcDNA3 plasmid only. Mock-infected cells were treated with culture medium. Cell lysates were prepared at 48 h post-infection and assayed for luciferase activity. (B) Cf2-Luc cells transfected with pcDNA3-CD4 alone or cotransfected with pcDNA3-CD4 and plasmids expressing CCR5, CX3CR1, RDC1, CCR7, CXCR1, CXCR2, CXCR3, C5aRC9, D2DRsC9, DARC, D6 or FMLP-R were infected with equivalent amounts of each HIV-1 virus. HIV-1 entry was measured as above. Data are represented as means from duplicate infections. Error bars represent standard deviations. Similar results were obtained in two independent experiments.

cells was strictly CD4-dependent (data not shown). We next tested whether the primary virus could utilize other GPCRs as coreceptors. Virus entry was detected in Cf2-Luc cells coexpressing CD4 and the promiscuous CC-chemokine receptor D6 (Nibbs et al., 1997), but not in cells coexpressing CD4 and CX3CR1, Rdc1, CCR7, CXCR1, CXCR2, CXCR3, complement 5a anaphylatoxin receptor (C5aRC9), dopamine receptor 2-short form (D2DrsC9), duffy antigen/receptor for chemokines (DARC), or formyl-methionine–leucine–phenylalanine receptor (FMLP-R) (Fig. 1B). Cell surface expression of these GPCRs was readily detected (M. Farzan and H. Choe, unpublished observations). Preincubation with zidovudine (AZT) abolished infection (data not shown). The broad and efficient usage of alternative coreceptors by the patient virus was unique by comparison to over 50 other primary HIV-1 viruses analyzed in similar assays (Gorry et al., 2001, 2002b; Gray et al., 2005; Lawson et al., 2004; Solomon et al., 2005 and data not shown). Thus, the patient virus demonstrates unusually broad and efficient usage of alternative coreceptors compared to other HIV-1 strains.

Replication kinetics

We examined the capacity of the primary virus to replicate in PBMC, CEMx174 cells, and monocyte-derived macrophages (MDM) (Fig. 2A). The ADA and NL4-3 viruses were used as positive controls for replication in PBMC; NL4-3 was used as a positive control for replication in CEMx174 cells; ADA, the R5 YU2 strain, and the X4 NDK strain (Ancuta et al., 2001; Gorry et al., 2001) were used as positive controls for replication in MDM. In PBMC, the primary virus replicated to low levels compared to NL4-3 and ADA, reaching peak levels of replication at day 10 post-infection compared to days 4 and 7 for NL4-3 and ADA, respectively. In CEMx174 cells, the primary virus reached peak levels of virus replication at day 24 post-infection compared to day 7 for NL4-3. In MDM, the primary virus replicated at low levels compared to ADA, YU2 and NDK. These results, together with experiments showing that the same primary virus stock had similar infectivity on Cf2-Luc cells coexpressing CD4 and CCR5 or CXCR4 compared to the control viruses (Fig. 1), suggest that the primary virus has reduced replication capacity in PBMC, CEMx174, and MDM compared to several laboratory and primary HIV-1 strains.

Sensitivity to CCR5 and CXCR4 inhibitors

The efficient usage of alternative coreceptors in transfected cells raised the possibility that infection of primary CD4+ cells by the primary virus might be mediated by alternative coreceptors. Therefore, sensitivity to small molecule inhibitors of CCR5 or CXCR4 (TAK-779 and AMD3100, respectively) was tested in PBMC and MDM. We first determined sensitivity to AMD3100 and TAK-779 in PBMC at a range of concentrations previously shown to be effective at inhibiting infection by other primary HIV-1 viruses (Gorry et al., 2002a). AMD3100 or AMD3100 and TAK-779 in combination abolished infection of

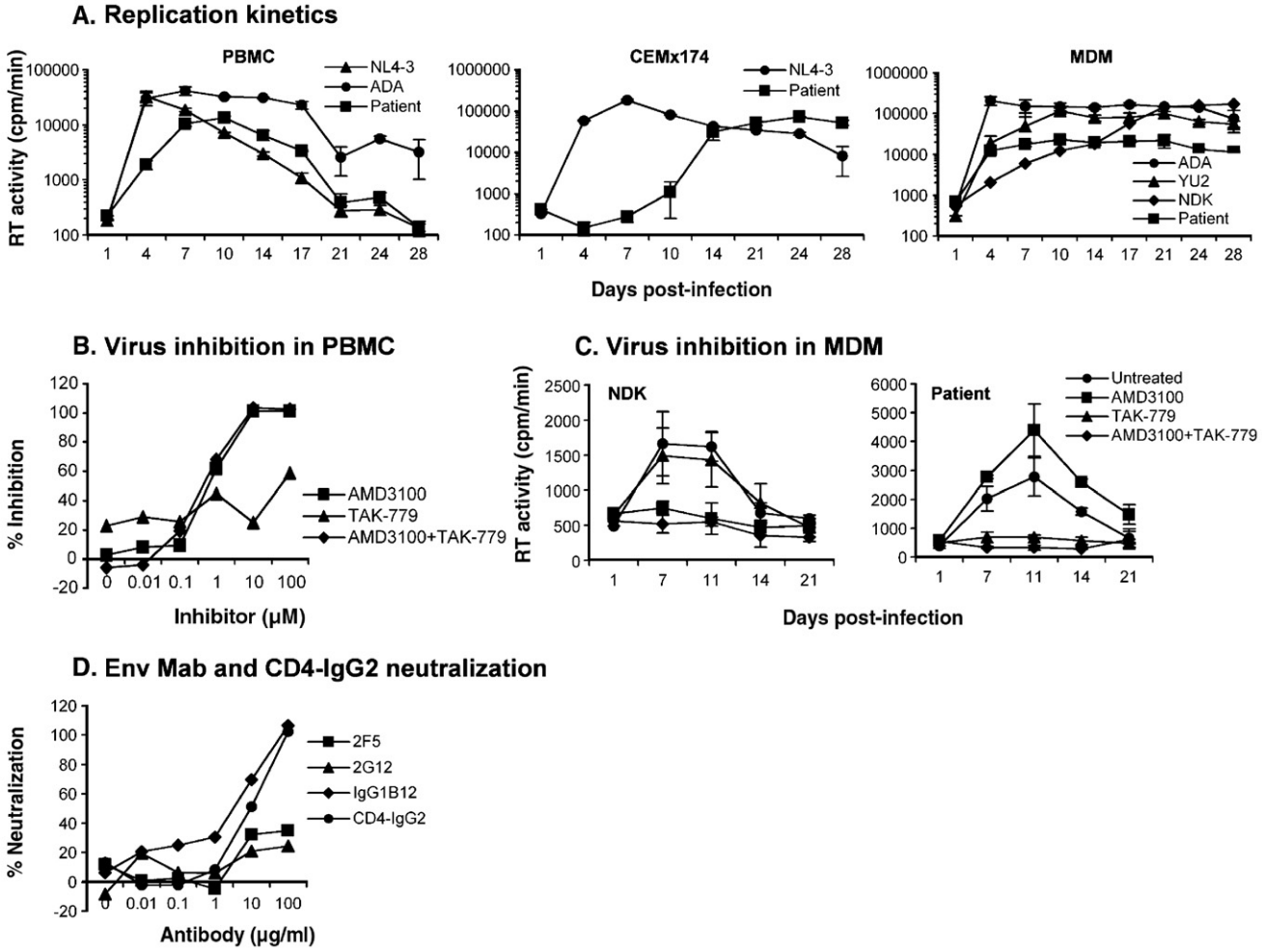

Fig. 2. Replication kinetics and sensitivity to coreceptor inhibitors and antibody neutralization. (A) PBMC, CEMx174 cells and MDM were infected with equivalent amounts of each HIV-1 virus, as described in Materials and methods, and cultured for 28 days. Virus production in culture supernatants was measured by RT assays. (B) PBMC were treated with concentrations of CCR5 (TAK-779) or CXCR4 inhibitors (AMD3100), or both inhibitors increasing 10-fold from 0.01 to 100 μM, and infected with the patient virus in the presence of each concentration of inhibitor. Virus production in culture supernatants at day 14 post-infection was measured by quantitation of soluble HIV-1 p24 Ag. Production of p24 Ag was calculated as a percentage of the amount produced in the absence of inhibitors, and then expressed as the percentage of inhibition relative to cultures containing no inhibitor. (C) MDM were treated with TAK-779 (100 nM), AMD3100 (1.2 μM) or both for 1 h prior to infection. Untreated cells contained no inhibitor. Cells were infected with equivalent amounts of HIV-1 NDK or the patient isolate and cultured for 21 days in the presence of each inhibitor. HIV-1 production in culture supernatants was measured by RT assays. (D) Virus stocks were treated with concentrations of MAbs or CD4-IgG2 increasing 10-fold from 0.01 to 100 μM for 30 min, and used to infect PBMC. Virus production in culture supernatants and calculation of percent neutralization was determined as per panel (B). Values shown are means from duplicate infections. Error bars represent standard deviations (A, C). Results are representative of two independent experiments using cells obtained from different donors.

PBMC at day 14 post-infection, whereas treatment with TAK-779 alone had only a modest inhibitory effect (Fig. 2B). Similar results were obtained at days 7 and 10 post-infection (data not shown). The 50% inhibitory concentration (IC_{50}) and IC_{90} of AMD3100 at day 14 post-infection was 0.65 μM and 5.5 μM, respectively, whereas the IC_{50} and IC_{90} of TAK-779 at day 14 was 58 μM and >100 μM, respectively. PBMC from a donor homozygous for the CCR5 Δ32 allele supported replication of the primary virus, which was completely abolished by AMD3100 (data not shown). These results demonstrate that the virus primarily uses CXCR4 for infection of PBMC. We then determined sensitivity to AMD3100 and TAK-779 in MDM at concentrations previously shown to completely inhibit infection of MDM or microglia (Gorry et al., 2001; Simmons et al., 1998) (Fig. 2C). AMD3100 abolished infection of MDM by NDK, but had no inhibitory effect on the primary virus. TAK-779 abolished infection of MDM by the primary virus, but had no inhibitory effect on NDK. AMD3100 and TAK-779 in combination abolished infection of MDM by either virus. These results demonstrate exclusive use of CCR5 for infection of MDM. Thus, despite broad coreceptor utilization in transfected cells, infection of PBMC and MDM was mediated only by CXCR4 and CCR5, respectively.

We previously showed that primary human adult astrocytes transduced with CD4 via an adenovirus vector and primary brain microvascular endothelial cells (BMVECs) that express low levels of CD4 can support infection by a subset of HIV-1, HIV-2 and SIV isolates in a CCR5- and CXCR4-independent manner (Willey et al., 2003). To further investigate whether the primary virus can use alternative coreceptors for virus entry in

primary human cells, we infected these cells with the primary virus, as previously described (Willey et al., 2003). No evidence of infection of CD4-expressing astrocytes or BMVECs by the primary virus was observed, whereas both cell types supported productive virus replication by the R5X4 HIV-1 viruses GUN-1v and HAN-2, the HIV-2 strain TER, and the SIV strain 17Efr (data not shown). These studies provide further evidence that infection of primary cells by the virus is unlikely to be mediated by alternative coreceptors.

Sensitivity to antibody neutralization

We next measured the sensitivity of the primary virus to neutralization by Env monoclonal antibodies (MAbs) and CD4-IgG2, as previously described (Trkola et al., 1995) (Fig. 2D). The neutralizing reagents were human MAbs 2F5 (Muster et al., 1994; Trkola et al., 1995), 2G12 (Trkola et al., 1995, 1996), IgG1b12 (Burton et al., 1991, 1994), and the tetrameric CD4-IgG2 molecule (Allaway et al., 1995). The virus was moderately sensitive to neutralization by IgG1b12 and CD4-IgG2, but showed a high level of resistance to neutralization by 2F5 and 2G12, similar to that of 2 other primary viruses studied in parallel (Fig. 2D and data not shown). Thus, the primary virus is relatively resistant to neutralization by Env MAbs and CD4-IgG2, similar to the majority of primary HIV-1 viruses.

Neutralization of heterologous viruses by the subject's plasma

We determined the ability of the subject's plasma (obtained in September 2001; see Supplementary Table 3) to inhibit infection of PBMC by 6 clade B and 2 clade A HIV-1 viruses as compared with HIVIG and MAbs IgG1b12, 2F5, and 2G12, as previously described (Mascola et al., 2002). The IC_{50}s and IC_{90}s are summarized in Table 1. The subject's plasma and HIVIG neutralized infection by 6 diverse clade B and 1 of 2 clade A HIV-1 viruses at plasma dilutions increasing 5-fold from 1:5 to 1:625, or HIVIG concentrations increasing 10-fold from 10 μg/ml to 10 mg/ml (Mascola et al., 2002). In contrast, the MAbs IgG1b12, 2F5 and 2G12, at concentrations increasing from 0.05 μg/ml to 50 μg/ml, neutralized infection by a subset of the viruses tested. Compared to plasma and sera from other HIV-1-infected individuals, the potency and breadth of the cross-neutralizing activity of the patient's plasma were in the top 10% of HIV-1-positive plasma/sera (J. Mascola, unpublished data). Together, these studies suggest that the subject's plasma has high levels of cross-neutralizing antibodies.

Characterization of full-length gp160 Env clones

The gp160 coding region of HIV-1 Env was cloned into the pCR3.1-Uni expression vector. Full-length, functional Env clones were identified by Western blot analysis of gp120/gp160 in transfected 293T cells and by fusion assays. Four Env clones that express distinct gp160 and gp120 proteins detected by Western blot analysis of transfected 293T cells (Fig. 4A) were sequenced (Fig. 3). Env gp160 amino acid sequences were uninterrupted in Env clones 6, 16 and 30, but contained a premature truncation of 4 amino acids in the gp41 cytoplasmic region in clone 12. The net charge of the V3 variable loops was +5 in all 4 Env clones. The Env clones contained an asparagine-rich insertion of 10 amino acids at positions 131 to 140 in the V1 variable region, which is unique among 207 clade B Envs screened in the Los Alamos database. Two similar asparagine-rich insertions of 11 and 6 amino acids were identified at positions 192 to 202 and 208 to 213, respectively, in the V2 variable region. These insertions result in a net gain of one potential N-linked glycosylation site in V1, and 3 potential N-linked glycosylation sites in V2. The total number of potential N-linked glycosylation sites in gp120 was 20 to 21 compared to 25 in the clade B consensus sequence.

To determine whether the Env clones are representative of the predominant HIV-1 variants in the viral quasispecies, fusion and single round entry assays were performed (Figs. 4B, C). 293T cells expressing each Env (Fig. 4A) were fused with Cf2-Luc cells cotransfected with CD4 and a coreceptor as described (Gorry et al., 2002a) (Fig. 4B). 293T cells expressing a non-functional Env or expressing the ADA, HXB2 or 89.6 Env were used as negative and positive controls, respectively. The

Table 1
Virus neutralization studies

Plasma or Ab		Clade B HIV-1						Clade A HIV-1	
		SF162	BaL	89.6	dBR07	aBL01	6101	UG031	RW020
Patient[a]	IC_{50}	255	220	261	553	90	105	187	32
	IC_{90}	42	31	64	256	18	27	10	7
b12[b]	IC_{50}	0.4	1.1	0.3	0.6	12	>50	>50	>50
	IC_{90}	2.7	12	5.5	22	>50	>50	>50	>50
2F5	IC_{50}	2.9	1.1	0.1	1.1	3.1	50	9	0.3
	IC_{90}	>50	>50	2	>50	>50	>50	>50	4
2G12	IC_{50}	17	0.1	>50	>50	>50	29	>50	0.6
	IC_{90}	>50	10	>50	>50	>50	>50	>50	5
HIVIG	IC_{50}	40	320	13	140	685	850	5420	2004
	IC_{90}	680	6590	430	2610	>10000	8700	>10000	6050

[a] IC_{50} and IC_{90} values of patient plasma neutralization are expressed as the reciprocal of plasma dilution required to inhibit 50 or 90%, respectively, of viral infection of CD8-depleted PBMC. The patient plasma was obtained in September, 2001 (see Supplementary Table 3).

[b] IC_{50} and IC_{90} values of Env MAbs and HIVIG are expressed as the concentration (μg/ml) required to inhibit 50 or 90%, respectively, of viral infection of CD8-depleted PBMC.

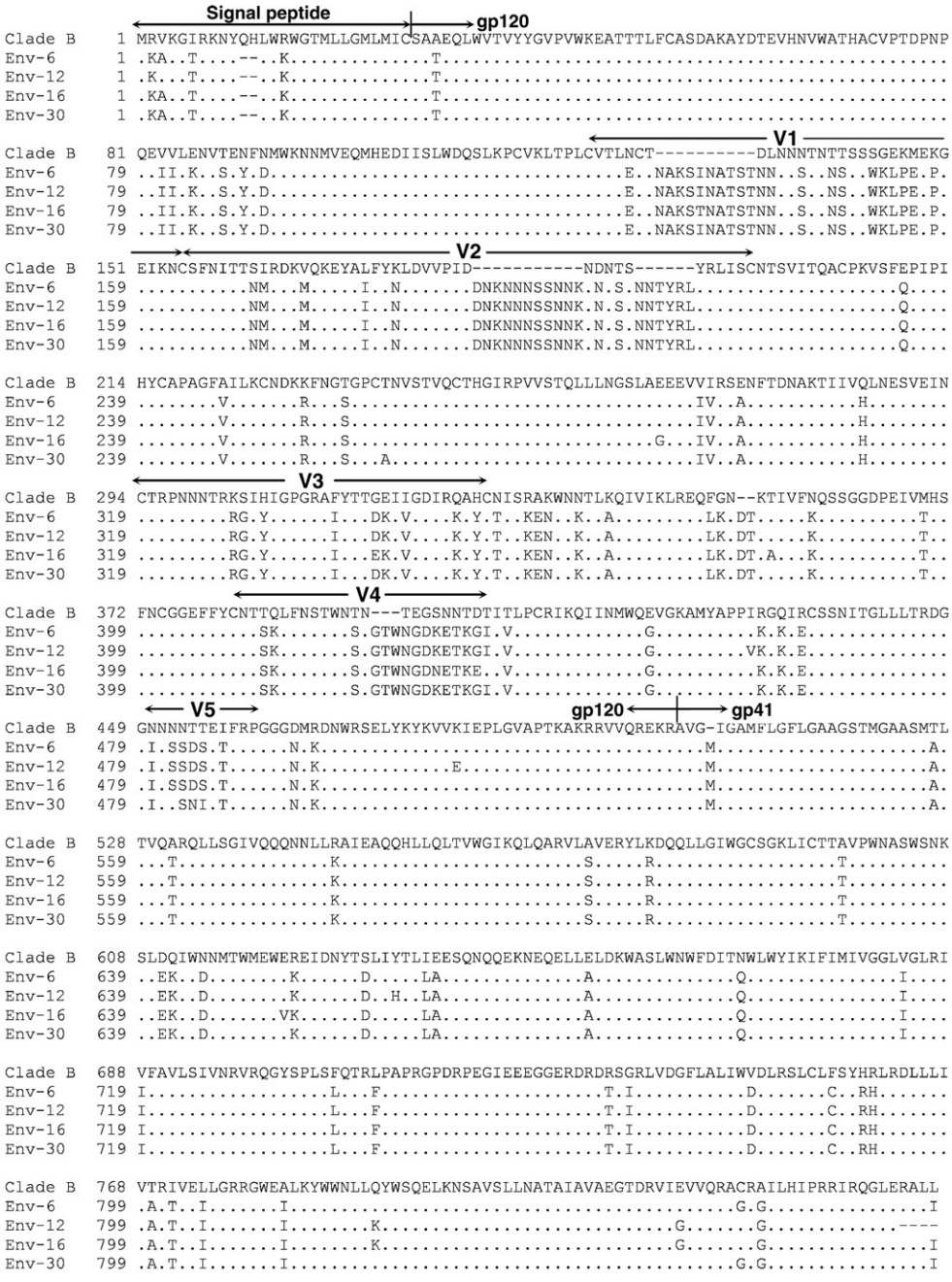

Fig. 3. Env amino acid sequences. Amino acid sequences were obtained from gp160 *env* genes cloned from genomic DNA of PBMC infected with the primary virus as described in Materials and methods. Amino acid alignments of Envs from the patient virus are compared to the clade B consensus sequence. Dots indicate residues identical to the clade B consensus and dashes indicate gaps.

primary Env clones functioned in fusion assays with Cf2-Luc cells coexpressing CD4 and CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33, Apj or D6. A low level of fusion was also observed with Cf2-Luc cells expressing CD4 only. We performed single round entry assays in Cf2th cells expressing CD4 only or coexpressing CD4 and each of the coreceptors (Fig. 4C). HIV-1 pseudotyped with Env clones 6 and 30 entered cells expressing CD4 alone, or coexpressing CD4 and CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33, Apj or D6. In contrast, HIV-1 pseudotyped with Env clones 12 and 16 entered cells coexpressing CD4 and CCR2b, CCR3, CCR5, CXCR4, Gpr15, Strl33 or Apj, but not cells expressing CD4 alone or coexpressing CD4 and CCR8, Gpr1 or D6. The levels of virus entry mediated by Env clones 12 and 16 were lower than those mediated by Env clones 6 and 30. Together, these results demonstrate broad coreceptor usage by 4 Env clones in fusion and single round entry assays, similar to the pattern of coreceptor usage of the primary virus isolate (Fig. 1).

Analysis of Env determinants contributing to broad coreceptor usage

To investigate mechanisms underlying the broad coreceptor usage of these primary Envs, we analyzed sequences of the V3

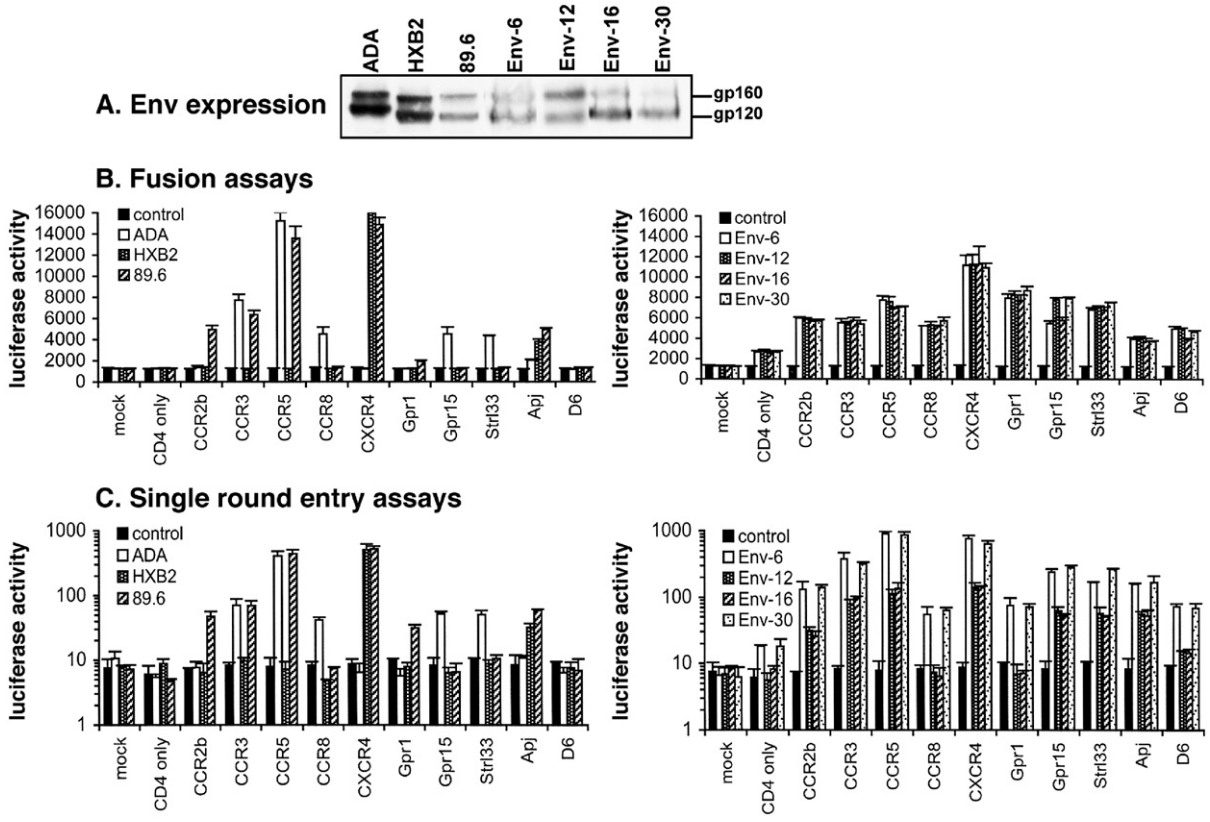

Fig. 4. Expression and function of full-length Env clones in cell–cell fusion and infection assays. (A) 293T cells were cotransfected with 15 μg pCR3.1 Env-expressing plasmid (Envs 6, 12, 16 and 30) or pSVIII Env-expressing plasmid (ADA, HXB2 and 89.6 Envs) and 2 μg pLTR-Tat. At 72 h post-transfection, cell lysates were analyzed by Western blotting using rabbit anti-gp120. The positions of gp160 and gp120 are indicated on the right. (B) 293T effector cells transfected with Env and Tat as above were mixed with Cf2-Luc cells transfected with pcDNA3-CD4 only or cotransfected with pcDNA3-CD4 and pcDNA3 expressing CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33, Apj or D6 and incubated at 37 °C for 12 h. Control 293T cells were transfected with ΔKS Env. Mock-transfected Cf2-Luc cells were transfected with pcDNA3 only. Cell lysates were then prepared and assayed for luciferase activity. (C) HIV-1 luciferase reporter viruses pseudotyped with each Env were generated and used to infect Cf2th cells transfected with pcDNA3-CD4 only or cotransfected with pcDNA3-CD4 and pcDNA3 expressing CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33, Apj or D6. Control virus was produced by pseudotyping with a non-functional Env (ΔKS Env). Cell lysates were prepared at 60 h post-infection and assayed for luciferase activity. Data are represented as means from duplicate wells in one experiment. Error bars represent standard deviations. Results are representative of two independent experiments, each performed in duplicate.

region, which modulates coreceptor usage (reviewed in Hartley et al., 2005), and the conserved coreceptor binding site on the Env core (Rizzuto et al., 1998) to identify amino acid variants that might influence Env–GPCR interactions. In the V3 region, patient Envs had Y308, a rare amino acid variant (1.5% of Clade B Envs, $n=23{,}470$) at a position implicated in modulating resistance to neutralizing antibodies (Zhang et al., 2002) and a small molecule CCR5 inhibitor (Kuhmann et al., 2004); I317, an amino acid that contributed to the ability of JR-CSF to use an N-terminal deletion mutant of CCR5 (Platt et al., 2001); D321 (or E321) and Y330, amino acid changes that affect the overall charge of V3 and thus may alter interactions with the tyrosine-sulfated N-terminal region of GPCRs; and T332, a change that results in the loss of a potential N-linked glycosylation site relative to the Clade B consensus (Fig. 5A). In the conserved coreceptor binding site on the Env core, patient Envs had K442 and E444 in the β22 strand near the base of V3, amino acid variants that could potentially affect electrostatic interactions at the Env–GPCR interface.

To investigate the contribution of these amino acid variants to broad coreceptor usage, we used site-directed mutagenesis to change each of these amino acids to the Clade B consensus residue in patient Env-30. The parental and mutant Envs were expressed at similar levels as determined by Western blotting (Fig. 5B, inset). The ability of Envs to use CCR5, CXCR4, and alternate coreceptors Gpr1, Gpr15, Strl33, and D6 was analyzed in cell-to-cell fusion and single round virus entry assays (Fig. 5B and data not shown). The Y308H mutation resulted in an increased ability to use CCR5, and to a lesser extent CXCR4, but reduced the capacity of Env-30 to use Gpr1, Gpr15, Strl33, and D6 ($p=0.001$, 0.02, 0.02, and 0.04, respectively; Student's t test). Additionally, a D321G mutation in Env-30 resulted in an increased utilization of CCR5, CXCR4, and D6 ($p=0.002$, 0.049, and 0.006, respectively), but decreased utilization of Gpr1 and Gpr15 ($p=0.006$ and 0.048, respectively). In contrast, a I317F mutation drastically reduced the capacity of Env-30 to use all coreceptors tested ($p<0.01$). A K442Q mutation resulted in a significant decrease in utilization of Gpr1 and Strl33 ($p=0.001$ and 0.01, respectively) and a minor decrease in utilization of D6 that did not reach statistical significance, while a E444R change reduced utilization of CXCR4 and Gpr15 ($p<0.01$). A Y330H mutation reduced the ability of Env-30 to

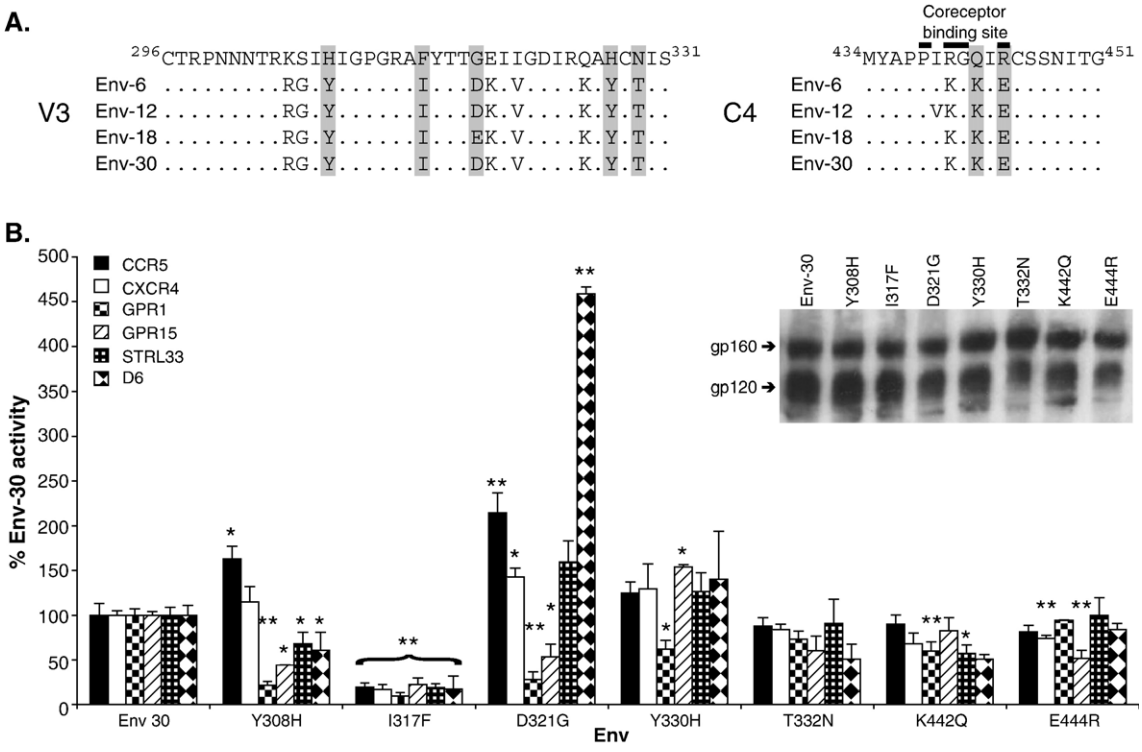

Fig. 5. Analysis of Env determinants contributing to broad coreceptor usage (A) V3 and C4 amino acid sequences of the Clade B consensus and patient Env clones were aligned with Clustal X. Dots represent amino acids identical to the Clade B consensus sequence. Residues important for CCR5 binding (Rizzuto et al., 1998) in the C4 region are indicated. Amino acid variants analyzed in mutagenesis studies are highlighted in gray. (B) HIV-1 luciferase reporter viruses pseudotyped with each Env were generated and used to infect Cf2th cells cotransfected with pcDNA3-CD4 and pcDNA3 expressing CCR5, CXCR4, Gpr1, Gpr15, Strl33, or D6. Control virus was produced by pseudotyping with ΔKS Env. Cell lysates were prepared at 60 h post-infection and assayed for luciferase activity. Data were normalized to parental Env-30 activity and are represented as means from two to three independent experiments, each performed in duplicate. Error bars represent standard error of the mean. *, $p<0.05$; **, $p<0.01$, Student's t test. Inset, 293T cells were cotransfected with 15 μg Env-expressing plasmids and 2 μg pLTR-Tat. At 72 h post-transfection, cell lysates were analyzed by Western blotting using goat anti-gp120. The positions of gp160 and gp120 are indicated on the left.

use Gpr1 ($p=0.04$), but enhanced utilization of Gpr15 ($p=0.01$) and had no significant effect on utilization of Strl33 and D6. A T332N mutation had no significant effect on entry efficiencies and patterns of coreceptor usage compared to the parental Env-30 (Fig. 5B). These results suggest that Y308, D321, and to a lesser extent K442 and E444, contribute to enhanced gp120 interactions with alternate coreceptors, and raise the possibility that I317 may be a compensatory change in patient Env-30.

Molecular modeling of V3 determinants

To elucidate a mechanism for enhanced gp120–coreceptor interactions in Env-30, we used Swiss PDB viewer to model the amino acids at positions 308, 317, and 321 on the JRFL gp120-CD4-X5 CD4i Ab crystal structure (2B4C) (Huang et al., 2005). The Clade B consensus amino acids at positions 308 and 317 in the V3 tip region are His and Phe, respectively (Fig. 6A). If the His at 308 is changed to Tyr, there is an increased potential for steric clashing when the aromatic ring of Phe is present at position 317 (Fig. 6B), whereas the smaller side chain of Ile at this position can accommodate the Tyr at 308 (Fig. 6C). This finding suggests that the loss of Env function in the I317F mutant may be due to the lack of a compensatory change at position 308. Consistent with this prediction, when Y308 is present in Clade B Envs ($n=360$), there is a decreased frequency of amino acids with bulky aromatic side chains such as Phe and Trp, but an increased frequency of amino acids with small hydrophobic chains such as Ile and Val at position 317 compared to Clade B Envs ($n=23{,}470$) (Table 2), suggesting that covariation may occur at positions 308/317 *in vivo*. In Envs with Y308 ($n=360$ Env sequences from 77 patients), I317 appeared in 23.9% ($n=86$ Env sequences from 11 patients) compared to 4.1% ($n=959$) of Clade B Envs in the database ($n=23{,}470$) (Table 2). At position 321, Gly may increase the flexibility of the V3 stem, allowing increased gp120 binding to GPCRs. An Asp at this position may decrease the flexibility of the stem, but may also enhance the ability of Env-30 to interact with some GPCRs that contain positive charges in the N-terminus, such as Gpr1 and Gpr15. In Envs with Y308, there is an increased frequency of charged amino acids at position 321 compared to Clade B Envs, raising the possibility that covariation may also occur at 308/321 (Table 2).

Analysis of Env interactions with attenuated CCR5 coreceptors

GPCRs are 7-transmembrane receptors with an extracellular, post-translationally modified acidic N-terminal region and three

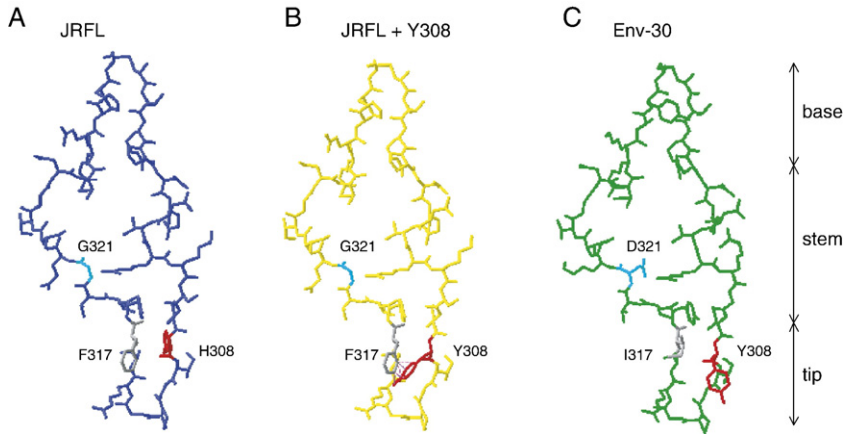

Fig. 6. Structural modeling of Env V3 determinants contributing to broad coreceptor usage. Swiss PDB viewer was used to model amino acid changes at positions 308, 317, and 321 on the V3 region of the JRFL gp120-CD4-X5 CD4i Ab crystal structure (2B4C) (Huang et al., 2005). JRFL V3 has a single amino acid change relative to the clade B consensus (N301Q). Red, position 308. Gray, position 317. Cyan, position 321. (A) V3 region of JRFL with H308, F317, and G321. (B) A Y308 change in JRFL results in steric clashing with F317 (dotted lines). (C) Changes Q301N, K305R, S306G, Y308H, F317I, G321D, E322K, I324V, Q328K, H330Y, and N332T were introduced in JRFL to produce a model of the V3 region of Env-30. Positions Y308, I317, and D321 are indicated.

extracellular loops (ECLs). The N-terminal region and ECL2 contain the major determinants for gp120 binding and coreceptor activity (Dragic et al., 1998; Farzan et al., 1998, 1999; Kuhmann et al., 1997; Lee et al., 1999; Olson et al., 1999; Rabut et al., 1998; Rucker et al., 1996). The N-terminus of CCR5 has Tyr sulfation modifications at several sites that are important for mediating HIV entry (Farzan et al., 1999). To determine whether amino acid variants in Env-30 influence interactions with the N-terminus or ECL2 regions of GPCRs, Cf2 cells expressing CD4 and either wild-type CCR5 (CCR5 (wt)), CCR5 (Y14N) which has a point mutation in the N-terminal region that changes a Tyr sulfation site important for mediating HIV-1 entry to an N-linked glycosylation site (Farzan et al., 1999; Kuhmann et al., 2000), CCR5 (Δ18) which contains a deletion of the first 18 amino acids of the N-terminal region, or CCR5 (G163R) which contains a point mutation in the transmembrane region associated with a conformational change in ECL2 that is detrimental to HIV-1 entry (Platt et al., 2001; Siciliano et al., 1999), were infected with viruses expressing wild-type or mutant Envs. Primary, macrophage tropic R5 viral isolates use CCR5 (Y14N) and CCR5 (G163R) at low efficiencies (1.5% and 10–20% compared to CCR5 (wt), respectively) (Kuhmann et al., 2000; Platt et al., 2001) and are unable to use CCR5 (Δ18) (Platt et al., 2005). In contrast to primary R5 Envs, Env-30 used CCR5 (wt) and CCR5 (G163R) with similar efficiencies (Fig. 7). Y308H and D321G had reduced utilization of CCR5 (G163R) relative to CCR5 (wt) compared to Env-30 (Fig. 7), suggesting that amino acid variants at these positions in patient Env-30 reduce dependence on the ECL2 region of CCR5. The Y308H, Y330H, K442Q, and E444R mutants used CCR5 (Y14N) for entry more efficiently compared to Env-30 (Fig. 7). None of the Envs could use CCR5 (Δ18) efficiently, consistent with the critical role of the N-terminal region of GPCRs in binding and entry (Dragic et al., 1998; Farzan et al., 1998, 1999; Kuhmann et al., 1997; Rabut et al., 1998; Rucker et al., 1996). However, the Y308H, D321G, T332N, and E444R mutations resulted in an increase in the ability of Env-30 to use CCR5 (Δ18), albeit at very low efficiencies. Cell surface expression levels of CD4 and CCR5 on transfected Cf2th cells were similar based on staining with CD4-FITC (RPA-T4) and CCR5-PE (2D7) and FACS analysis (data not shown). Together, these results suggest that Y308 and D321 reduce dependence on the ECL2 region of CCR5, while these residues along with Y330, K442, and E444 increase dependence on the

Table 2
Frequency and co-variation of unusual amino acid variants *in vivo*

Position	Amino Acid	Clade B Envs (n=23470)[a]		Envs with Y308 (n=360)		
		Frequency (%)[b]	n	Frequency (%)	n	Significance[c]
317	F	78.6	18446	23.6	85	**
	W	6.4	1500	0.6	2	**
	L	5.1	1194	5.0	18	
	I	4.1	959	23.9	86[d]	**
	V	2.7	644	45.3	163	**
	Y	1.8	411	0.0	0	**
	Other	1.3	316[e]	1.7	6[f]	
321	G	79.8	18740	36.7	132	**
	E	4.1	970	19.7	71	**
	D	2.8	667	7.5	27	**
	K	2.0	460	26.9	97	**
	R	1.7	389	8.1	29	**
	Other	9.6	2244[g]	1.1	4[h]	

[a] V3 sequence alignment was obtained from the Los Alamos database in October 2005. Sequence data for Envs with Y308 are from 77 patients.
[b] Frequency was calculated as [(n/total number of sequences)×100].
[c] Differences between groups were significant as calculated by Fisher's Exact Test. **, $p<0.005$.
[d] Envs with Y308/I317 are from 11 patients.
[e] Other indicates amino acids M, S, K, R, C, Q, H, P, T, G, A, D, and E (in descending order of frequency), or could not be determined.
[f] Other indicates amino acids M, S, K, E, and P (in descending order of frequency).
[g] Other indicates amino acids T, A, Q, N, S, I, and V (in descending order of frequency), a gap in the alignment, or could not be determined.
[h] Other indicates amino acid Q or could not be determined.

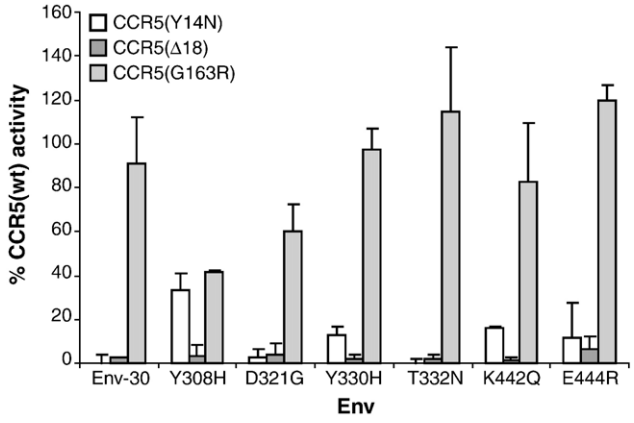

Fig. 7. Env interactions with attenuated CCR5 coreceptors. HIV-1 luciferase reporter viruses pseudotyped with each Env were generated and used to infect Cf2th cells cotransfected with pcDNA3-CD4 and pcDNA3 expressing CCR5 (Y14N), CCR5 (Δ18), or CCR5 (G163R). Control virus was produced by pseudotyping with ΔKS Env. Cell lysates were prepared at 60 h post-infection and assayed for luciferase activity. Results for each mutant CCR5 were normalized to levels of luciferase activity in cells expressing CCR5 (wt) for each Env and are represented as means from two independent experiments, each performed in duplicate. Error bars represent standard deviations.

CCR5 N-terminus compared to clade B consensus residues at these positions.

Discussion

In this study, we isolated and characterized an unusual HIV-1 strain from the blood of an asymptomatic individual who was heterozygous for the CCR5 Δ32 allele and had reduced levels of CCR5 expression. The primary virus is dual-tropic and exhibits unusually broad and efficient utilization of alternative coreceptors. The repertoire of alternative coreceptors used is greater than that of any previously described HIV-1 virus, and more closely resembles that of some SIV and HIV-2 viruses than that of HIV-1 viruses (Morner et al., 1999; Rucker et al., 1997; Siciliano et al., 1999). However, in contrast to some SIV and HIV-2 strains that can infect cells in the absence of CD4 (Reeves et al., 1997, 1999), CCR5- and CXCR4-mediated entry by the primary virus is strictly CD4-dependent. Previous studies suggest that expanded use of alternative coreceptors is associated with rapid HIV-1 disease progression (Bjorndal et al., 1997; Connor et al., 1997). However, the virus we described here was isolated from an individual who was asymptomatic for 18 years and had slow disease progression, suggesting that expanded tropism of HIV-1 can occur in some individuals who do not have rapid disease progression.

Despite efficient usage of many alternative coreceptors in transfected cells, the virus exclusively used CXCR4 or CCR5 to enter primary T cells and monocyte-derived macrophages, respectively. A similar pattern of coreceptor preference for infection of these primary cells was described for the R5X4 89.6 isolate. CD4-expressing astrocytes and BMVECS were not infected by the primary virus, whereas both cell types supported infection by other unusual HIV-1 and HIV-2 strains. Thus, infection of primary cells was mediated only by CXCR4 and CCR5.

In addition to using CCR2b, CCR3, CCR5, CCR8, CXCR4, Gpr1, Gpr15, Strl33 and Apj, the patient virus also utilized the promiscuous CC-chemokine receptor D6 (Nibbs et al., 1997). Only two previous studies demonstrated HIV-1 entry mediated via D6 (Choe et al., 1998; Neil et al., 2005). D6 is expressed on lymphatic endothelial cells, but not on peripheral blood cells or vascular endothelial cells (Nibbs et al., 2001). In addition to entry mediated by D6, entry mediated by an endogenous canine coreceptor was demonstrated, albeit at very low levels. This property was not observed for over 50 other primary HIV-1 isolates tested in similar assays (data not shown). The identity of the endogenous canine coreceptor is unknown. However, entry via this coreceptor was not inhibited by AMD3100, suggesting it most likely is not CXCR4 (data not shown).

CCR5 cell surface expression levels were very low on the study subject's PBMC compared to other CCR5 Δ32 heterozygotes (Cohen et al., 1997; de Roda Husman et al., 1999). Previous studies demonstrated an increased frequency of R5X4 viruses in CCR5 Δ32 heterozygotes compared to CCR5 wt/wt individuals (Lathey et al., 2001; Zhang et al., 1998). We speculate that structural features that enhance interaction of the primary virus Env described herein with conserved structural elements in GPCRs may have resulted from adaptive evolution in the setting of very low levels of CCR5 cell surface expression. However, validation of this hypothesis would require longitudinal analysis of Env sequences in the patient's plasma and their functional characterization. The primary virus did not show enhanced sensitivity to antibody neutralization, but the subject's plasma had potent neutralizing activity against a wide variety of HIV-1 strains. This finding raises the possibility that the Env from the subject's virus may express epitopes that are immunogenic and neutralization functional, and may be responsible for at least some of the cross-reactive neutralizing activity of his plasma. A better understanding of these epitopes and their relationship to expanded coreceptor usage may provide insights relevant for improving HIV-1 immunogenicity and the elicitation of anti-HIV-1 neutralizing antibodies.

The broad usage of alternate coreceptors suggests that the Env of this primary virus has unique structural features that enhance interaction with conserved structural elements in GPCRs. In the V3 region, patient Envs had the unusual amino acid variants Y308 (present in 1.5% of Clade B Envs, $n=23{,}470$), I317 (4.1%), and D321 (2.8%). Y308H and D321G mutations increased the capacity of Env-30 to use CCR5 and to a lesser extent CXCR4, but decreased the utilization of alternate coreceptors, and an I317F mutation drastically decreased the capacity of the Env to use any coreceptor. Structural modeling suggests that a Y308 change in the JRFL crystal structure results in steric clashing with F317 (the clade B consensus residue), whereas the smaller side chain of Ile at this position is accommodated. Thus, I317 is likely to be a compensatory change for Y308. D321 may enhance electrostatic interactions with the positively charged N-terminus of CCR5 and other GPCRs. These observations, together with evidence for covariation of amino acids at positions 308/317 and 308/321 *in vivo* based on database analysis, suggest that Y308, I317, and D321 may act cooperatively to enhance Env–GPCR interactions. Env-30 used

CCR5(G163R), a mutant CCR5 which does not affect entry of SIV_{mac251} but reduces entry of primary macrophage tropic isolates such as ADA and SF162 (Kuhmann et al., 1997), for entry at levels comparable to those mediated by CCR5 (wt). This finding is consistent with the promiscuous coreceptor usage of the patient isolate and Env-30, which resembles that of SIV or HIV-2 (Edinger et al., 1998; Morner et al., 1999; Reeves et al., 1997, 1999; Rucker et al., 1997). In addition to enhancing utilization of alternative coreceptors, Y308 and D321 reduced the dependence of Env-30 on the ECL2 region of CCR5, while introducing the clade B consensus residues H308, H330, K442, and E444 resulted in reduced dependence on the CCR5 N-terminus. These findings are consistent with a proposed model in which the tip of V3 interacts with ECL2 of GPCRs, while the more conserved stem and base of V3 interact with the N-terminal region (Huang et al., 2005; Xiang et al., 2005). K442 and E444 in the C4 region may work in concert with the changes in V3 by facilitating interactions with the highly acidic N-terminal region of CCR5 and other GPCRs. Changes in other regions of Env, such as those in the V1/V2 region that cooperatively modulate coreceptor usage with V3 (Nabatov et al., 2004; Pastore et al., 2006; Sullivan et al., 1993), may further enhance Env–coreceptor interactions. Based on our results, we propose that changes in the V3 region of Env-30 cooperatively enhance interactions with conserved structural elements in GPCRs, while changes in the base of V3 and the C4 region may enhance electrostatic interactions with the N-terminus of GPCRs.

Analysis of patient Env sequences revealed a unique insertion of 10 amino acids in the V1 region, and two asparagine-rich insertions of 11 and 6 amino acids in the V2 region. Similar extensions in the V2 region have been associated with slow HIV-1 progression or nonprogression (Shioda et al., 1997; Wang et al., 2000). The V1 insertion in this subject is unusual compared to other clade B Envs, and the V1/V2 region is longer than that of other Envs in the database. Changes in Env that reposition the V1/V2 loops can increase exposure of the coreceptor binding site (Kolchinsky et al., 2001). These findings raise the possibility that the unusually long V1/V2 region may reposition the V1/V2 loops, resulting in a conformation that enhances interaction of gp120 with conserved elements in GPCRs. Conserved elements in GPCRs important for HIV-1 entry include the tyrosine-rich sulfated regions in the N-terminus, which are important for CCR5-mediated HIV-1 entry (Farzan et al., 1999). GPCRs that mediate entry by the primary virus have many tyrosine residues within the N-terminus that are probably sulfated in close proximity, similar to the pattern of sulfated tyrosine residues in CCR5. Thus, the tyrosine-rich region in the N-terminus is likely to be one of the conserved elements in GPCRs involved in HIV-1 binding and virus entry.

The subject's slow disease progression despite harboring a dual-tropic HIV-1 strain with highly expanded coreceptor usage may reflect additional viral and/or host factors that can influence HIV-1 progression. The primary virus had attenuated replication kinetics in PBMC, CEMx174, and MDM compared to several laboratory strains (Fig. 3A) and other primary viruses (Gorry et al., 2001). However, we did not determine whether the virus studied here was one of the majority sequences present in the patient. Mutations in the *nef* gene can cause viral attenuation and reduced pathogenicity of HIV-1 strains (Deacon et al., 1995; Kirchhoff et al., 1995). However, we found no deletions or other obvious defects in *nef* genes cloned from the primary virus, which were fully functional for CD4 and MHC-I down-regulation (K. Agopian and D. Gabuzda, unpublished data). Further studies are required to determine whether the primary virus contains any mutations in other viral genes that might be associated with slow disease progression, and whether it is a major variant in the patient viral quasispecies in vivo.

In summary, we describe an unusual dual-tropic HIV-1 strain with highly expanded coreceptor usage isolated from an asymptomatic individual who was heterozygous for the CCR5 Δ32 allele and had low CCR5 cell surface expression. In this subject, highly expanded coreceptor usage of HIV-1 occurred without progression to AIDS, suggesting that R5X4 HIV-1 strains with broadened coreceptor usage can be harbored by some individuals without rapid disease progression. Our results suggest that changes in the V3 and C4 regions, possibly a consequence of adaptive evolution in the setting of very low levels of CCR5 expression and viral escape from neutralizing antibodies, act cooperatively to broaden coreceptor usage by enhancing interactions with conserved structural elements in GPCRs. These results lead to a better understanding of HIV Env–GPCR interactions and provide insights that may facilitate development of vaccines and therapeutics that target virus entry.

Materials and methods

Cells

Peripheral blood mononuclear cells were purified from blood of healthy HIV-1-negative donors by Ficoll-Hypaque density gradient centrifugation, stimulated with 2 μg/ml phytohemagglutinin (PHA) for 3 days and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 20 U/ml interleukin-2 (IL-2; Boehringer Mannheim, Germany). CD8+ T cells were depleted by magnetic separation with anti-CD8-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). Monocyte-derived macrophages were purified from PBMC by plastic adherence and cultured for 5 days in RPMI 1640 medium supplemented with 10% (v/v) human AB+ serum and 12.5 ng/ml M-CSF. Cf2-Luc cells (Etemad-Moghadam et al., 2000), derived from the Cf2th canine thymocyte cell line (Choe et al., 1996), stably express the luciferase gene under the control of the HIV-1 LTR. Cf2-Luc cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, and 0.7 mg/ml G418. Cf2th cells were cultured in the same medium without G418. CEMx174 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS.

Isolation of HIV-1

HIV-1 was isolated from patient PBMC by coculture with CD8-depleted donor PBMC as described previously (Gorry et al., 2001). Briefly, 2×10^6 patient cells were added to

5×10^6 CD8-depleted PBMC from a normal uninfected donor, incubated at 37 °C for 1 h, and then cultured in 10 ml growth media containing 20 U/ml IL-2. Fifty percent media changes were performed twice weekly. Five million fresh PHA-activated, CD8-depleted PBMC from a different donor were added at every second media change. Supernatants were tested for reverse transcriptase (RT) activity using [^{3}H]dTTP incorporation. Supernatants testing positive for RT were filtered through 0.45 μm filters and stored at −80 °C.

Coreceptor usage

To determine coreceptor usage by the patient HIV-1 isolate, Cf2-Luc cells were cotransfected with 10 μg of plasmid pcDNA3-CD4 and 20 μg of plasmid pcDNA3 containing CCR2b, CCR3, CCR5, CCR8, CXCR4, CX3CR1, Gpr1, Gpr15, Strl33, Apj, CX3CR1, Rdc1, CCR7, CXCR1, CXCR2, CXCR3, C5aRC9, D2DrsC9, DARC, D6 or FMLP-R using the calcium phosphate method, and infected 48 h later by incubation with 10,000 ^{3}H cpm RT units of HIV-1 in the presence of 2 μg/ml polybrene as described previously (Gorry et al., 2001). Mock-infected cells were treated with culture medium. Cf2-Luc cells mock-transfected or transfected with pcDNA3-CD4 alone were used as negative controls. After overnight infection, virus was removed and the cells were cultured for an additional 48 h prior to lysis in 200 μl cell lysis buffer. Cell lysates were cleared by centrifugation, and assayed for luciferase activity according to the manufacturers' protocol (Promega).

HIV-1 replication kinetics

Five million PHA-activated PBMC were infected by incubation with 50,000 ^{3}H cpm RT units of virus supernatant in a volume of 2 ml for 3 h at 37 °C. Virus was then removed and PBMC were washed 3 times with PBS and cultured in media containing 20 U/ml IL-2 for 28 days. Monocyte-derived macrophages were isolated from PBMC by plastic adherence and allowed to mature for 5 days prior to seeding in 6-well tissue culture plates at approximately 90% confluence. Virus equivalent to 50,000 ^{3}H cpm RT units in a volume of 2 ml was allowed to adsorb to the cell monolayers for 3 h at 37 °C. Virus was then removed and cells were rinsed 3 times with PBS prior to addition of 2 ml culture medium. Five million CEMx174 cells were infected by incubation with 50,000 ^{3}H cpm RT units of virus supernatant in a volume of 2 ml for 3 h at 37 °C. Virus was then removed and cells were washed 3 times with PBS and cultured for 28 days. Fifty percent media changes were performed twice weekly and supernatants were tested for HIV-1 by RT assays.

Virus inhibition studies

The effects of the coreceptor inhibitors TAK-779 (Baba et al., 1999) and AMD-3100 (Donzella et al., 1998; Schols et al., 1997) on virus replication in PBMC were assayed as described elsewhere (Gorry et al., 2002a; Trkola et al., 1998). Briefly, PBMC were incubated for 30 min with a range of concentrations of each inhibitor (0.01 to 100 μM) prior to infection with the patient virus isolate. Virus replication was measured by production of HIV-1 p24 antigen in culture supernatants for 14 days (Trkola et al., 1995). The production of p24 antigen in the presence of an inhibitor was expressed as a percentage of the amount produced in control cultures containing no inhibitor. For virus inhibition studies in MDM, cells were preincubated with 100 nM TAK-779 or 1.2 μM AMD3100 for 1 h prior to infection with HIV-1 isolates containing the same concentration of inhibitor, as described previously (Gorry et al., 2001). Infected cells were cultured for 21 days in the presence of each inhibitor. Fifty percent media changes were performed weekly and supernatants were tested for HIV-1 by RT assays.

Neutralization assays

Human monoclonal antibodies (MAb) against HIV-1 gp120 (IgG1b12 and 2G12) and gp41 (2F5), the tetrameric CD4-immunoglobulin (CD4-IgG2) molecule, and purified polyclonal anti-HIV immunoglobulin (HIVIG) have been described previously (Allaway et al., 1995; Burton et al., 1991, 1994; Mascola et al., 2002; Muster et al., 1994; Trkola et al., 1995, 1996). Neutralization of replication of the patient virus isolate in PBMC was assessed as described previously (Trkola et al., 1995). Briefly, virus was incubated for 30 min with a range of concentrations of each Mab or CD4-Ig2 (0.01 to 100 μg/ml) prior to infection. Virus replication and calculation of percent neutralization were measured as described above. Neutralization of heterologous viruses by the subject's plasma was assessed as described by Mascola et al. (2002).

PCR amplification, HIV-1 Env cloning and sequence analysis

Genomic DNA was extracted from PBMC infected with the patient virus isolate using the DNeasy DNA extraction kit (Qiagen). Full-length *Env* genes were amplified from genomic DNA with RTth XL polymerase and nested primers using hot start AmpliWax PCR Gem 50 (Applied Biosystems), as described previously (Gorry et al., 2002a). Env PCR-product DNA was gel purified and cloned into pCR3.1-Uni (Invitrogen). Functional full-length Env clones were identified by Western blot analysis of gp120/gp160 in transfected 293T cells and by fusion assays. Env clones were sequenced using a model 3100 Genetic Analyzer (Applied Biosystems). Y308H, I317F, D321G, Y330H, T332N, K442Q, and E444R mutant Env plasmids were created by PCR-based mutagenesis and changes were verified by DNA sequencing.

Western blot analysis

For analysis of Env expression, 293T cells were transfected with 15 μg of different pCR3.1 Env clones, or 15 μg pSVIII plasmid expressing ADA, HXB2 or 89.6 Env plus 2 μg pLTR-Tat plasmid. At 72 h after transfection, cells were rinsed twice in PBS and resuspended in 400 μl of ice-cold lysis buffer for 20 min, followed by centrifugation at 15,300×*g* for 10 min to remove cellular debris. Cell lysates were separated in 8.5%

SDS-PAGE gels, and analyzed by Western blotting using rabbit anti-gp120 polyclonal antisera (American Biotechnologies Inc.) or goat anti-gp120 polyclonal antisera (NIH AIDS Research and Reference Reagent program). Env proteins were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-goat immunoglobulin G antibodies and enhanced chemiluminescence (Perkin Elmer).

Fusion assays

293T cells (1×10^5) cotransfected with 15 μg Env-expressing plasmid and 2 μg pLTR-Tat were mixed with Cf2-Luc cells (1×10^6) that had been cotransfected with 10 μg pcDNA3-CD4 and 20 μg pcDNA3 expressing an alternative coreceptor as indicated, then incubated at 37 °C in 0.75 ml culture medium. Mock-transfected Cf2-Luc cells were transfected with pLTR-Tat only. Control 293T cells were cotransfected with pLTR-Tat and a non-functional Env (pSVIII-ΔKS Env). Twelve hours later, cells were harvested and assayed for luciferase activity as described above.

Single round entry assays

An Env complementation assay was used to quantitate HIV-1 entry as described (Choe et al., 1996). Briefly, recombinant HIV-1 luciferase reporter viruses were generated by cotransfection of 293T cells by the calcium phosphate method with 16 μg of pNL4-3env$^-$ Luc, which contains an HIV-1 provirus with a deletion in the *env* gene and a replacement of the *nef* gene with a luciferase gene, and 6 μg of pCR3.1Env or pSVIIIEnv plasmid. Cf2th cells intended for use as target cells were cotransfected with 10 μg pcDNA3-CD4 and 20 μg pcDNA3 expressing an alternative coreceptor as indicated. Plasmids expressing CCR5 (Y14N), CCR5 (Δ18), and CCR5 (G163R) were kindly provided by D. Kabat. Approximately 48 h after transfection, these cells were infected by incubation with 20,000 ^{3}H cpm RT units of recombinant luciferase reporter viruses. Reporter viruses pseudotyped with a non-functional Env (pSVIII-ΔKSenv) were used as negative controls. Sixty hours later, cells were harvested and assayed for luciferase activity.

Nucleotide sequence accession numbers

Nucleotide sequences were submitted to GenBank (accession numbers AY624304 through AY624307).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.11.025.

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