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# Effect of a Single Amino Acid Substitution in the V3 Domain of the Human Immunodeficiency Virus Type 1: Generation of Revertant Viruses To Overcome Defects in Infectivity in Specific Cell Types

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**Proviral clones of human immunodeficiency virus type 1 which contained single amino acid changes in the envelope V3 region were constructed. PCR amplification of Sup-T1 T cells transfected with one such mutant, G312T, revealed low levels of virus that resulted in the generation of a revertant virus, in which an alanine replaced the threonine residue at amino acid 312. The revertant virus (rA312) was fully infectious in Sup-T1 cells but lacked the ability to infect AA5 cells. The presence of a second mutation in a subsequent revertant virus (rR306), in which arginine was substituted for serine at amino acid 306 within the V3 loop, restored the ability of the mutated virus to infect AA5 cells. Our data highlight the importance of the V3 loop in defining virus tropism for specific cell types in culture and further suggest that a degree of interplay exists among V3 loop residues that helps maintain or control its biological function of the virus.**

The mature form of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein comprises two major regions: the extracellular (gp120) and the transmembrane (gp41) domains. The gp120 glycoprotein binds with high affinity to CD4, the cellular receptor for HIV-1 (5, 18, 20), and antibodies that prevent the CD4-gp120 interaction are able to block HIV-1 infection (5, 18, 23, 38). A principal neutralizing determinant within gp120 has been mapped to an invariant cysteine loop, termed the V3 loop, which encompasses amino acid residues 296 to 331 of the BH10-specific envelope sequence (10, 16, 17, 22, 27, 30). Virus infectivity is effectively blocked by neutralizing antibodies that recognize V3 loop determinants in a manner that does not interfere with gp120 binding to CD4 (21, 34). Neutralizing antibody determinants have also been mapped to regions of gp41 (25). Regions within the amino terminus of gp41 have also been implicated in the process of virus-mediated cell-to-cell fusion (2, 43). Therefore, it appears that a complex series of interactions involving both gp120 and gp41 may be necessary not only to promote virus entry into target cells but also to elicit a neutralizing antibody response from the host.

The high degree of amino acid heterogeneity found within the V3 loop region among different HIV-1 isolates has been well-documented (19). This region is implicated in defining the cellular host for the virus, i.e., monocytes versus lymphocytes (7, 13, 32, 41, 42). Amino acid changes within the V3 loop which occur in vivo have been associated with a switch in the ability of virus isolates to induce syncytia in peripheral blood mononuclear cells or the T-cell line MT-2 (8, 40). This switch from non-syncytium-inducing (NSI) to syncytium-inducing (SI) variants occurs in 50% of HIV-1-seropositive individuals progressing to AIDS. In terms of cell tropism, NSI variants tend to be more monocytotropic, while SI variants appear to be lymphotropic (8). The most extensive variation associated with this phenomenon is found in the regions flanking the conserved tetrapeptide, GPGR, located at the tip of the V3 loop (4, 40).

Site-specific mutagenesis has been previously used to introduce mutations within the GPGR tip sequence of the V3 loop (14, 15, 26, 33). Many of these mutants were found to be defective; however, in select cases involving mutagenesis of the proline residue, mutant proviral clones produced virions with altered tropism and/or neutralization properties (15, 39). In this study, we describe the generation of a revertant virus from an otherwise defective proviral mutant clone. Comparison of this revertant virus and a subsequently isolated second revertant with the wild-type virus resulted in the identification of altered patterns of infectivity within different cell types.

The majority of mutations were introduced into the chimeric proviral clone (HXB2/10), which is composed of the HXB2 viral genome whose 2.7-kb *Sal*I-*Bam*HI envelope region (nucleotides [nt] 5366 to 8052) has been replaced with the corresponding region from the BH10 strain of HIV-1. The amino acid sequence of the gp120 proteins from the BH10 and HXB2 strains of HIV-1 differ by eight amino acids, only one of which occurs within the V3 loop. Construction of the 10/G312T mutant, in which the glycine at amino acid 312 of the envelope protein was changed to threonine, has been previously described (14). In select cases, V3 loop mutations were also introduced into the HXB2 proviral clone (HXB2). Specific mutations were introduced into the V3 loop region from either the BH10 or the HXB2 strain by a two-step oligonucleotide-directed mutagenesis method using PCR as described by Smith et al. (35). Typically in step 1, two separate PCRs were performed: reaction A, using the wild-type primer A3 (5′ ACTGCTGTTAAATGGCAGT, nt 6575 to 6594) and a variable mutant primer, MP2 (3′-5′ complementary strand of MP1; see below), and reaction B, using the wild-type primer B4 (5′ TCACTTCTCCAATTGTCCCT, nt 7227 to 7246) and a mutant primer, MP1 (5′-3′; MP1 sequences for specific mutants are listed below). In each of these reactions, 20 ng of template DNA consisting of the 2.7-kb *Sal*I-*Bam*HI region

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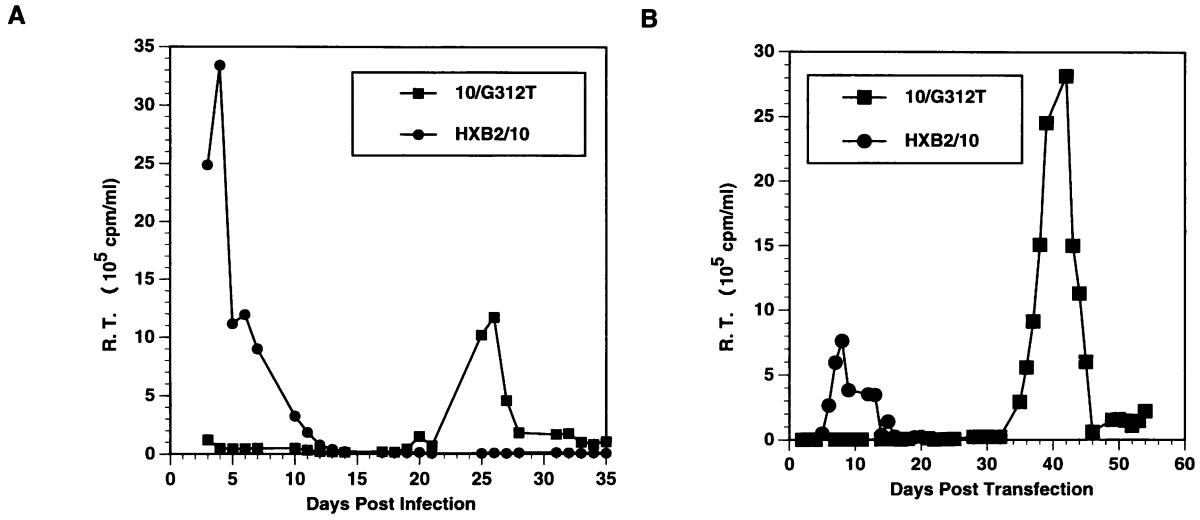


FIG. 1. (A) Infectivity profiles of 10/G312T HIV-1 envelope mutant and HXB2/10 wild-type viruses generated by transfection of proviral DNA into Cos-1 cells and cocultivation with Sup-T1 cells. RT levels in culture supernatants were measured daily for 35 days postinfection. (B) Infectivity profiles of 10/G312T HIV-1 envelope mutant and HXB2/10 wild-type viruses generated by transfection of proviral DNA directly into Sup-T1 cells. RT levels were measured daily in culture supernatants for 50 days posttransfection.

from either HXB2 or BH10 envelope was amplified in 10 mM Tris (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–200 μM each deoxynucleoside triphosphate (dNTP)–1.0 μM each primer–2 U of *Taq* polymerase in a final volume of 100 μl. The first 5 cycles of PCR amplification proceeded at 94°C for 2 min, 40°C for 1.5 min, and 72°C for 2 min; the remaining 30 cycles continued at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min. At the end of the last cycle, the reaction was extended at 72°C for 10 min. In step 2, 100-ng aliquots of gel-purified fragments from step 1 were mixed together, denatured by boiling for 5 min, and allowed to anneal at room temperature for 30 min. The ends were then filled in with Klenow fragment in a reaction mixture containing 40 mM $KPO_4$ (pH 7.5), 6.6 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 mM dATP, 250 μM dNTP, and 25 U of Klenow fragment. The reaction mixture was incubated for 1 h at room temperature and then overnight at 12°C. The 670-bp product was then amplified by PCR using wild-type fragments A3 and B4 with the cycling parameters described above for step 1. The fragment produced during step 2 was cut with *Bgl*II, and the resulting 580-bp fragment was subcloned into either a pBR322-based vector containing the 2.7-kb *Sal*I-*Bam*HI fragment of the BH10 genome or a pGEM5Zf+ vector (Promega Biotec) containing the 2.7-kb *Sal*I-*Bam*HI envelope fragment of HXB2. The final cloning step involved the insertion of a 2.7-kb mutant-bearing *Sal*I-*Bam*HI fragment into the viral vector pHXB2gpt (28). In addition to specific amino acid codon changes, silent mutations were included to further distinguish these clones from the wild-type sequence as well as to insert unique recognition sites for restriction enzymes. Mutations were confirmed in the viral vector clones by DNA sequencing and by restriction enzyme digestion (data not shown). The specific mutant clones generated were as follows (the mutant codons are underlined in the MP1 sequences): 10/G312A, 5′ ATC CAG AGA GCA CCA GGG AGA GCA (nt 6726 to 6749); 10/G312A/S306R, 5′ AAA CGA ATT CGT ATC CAG AGA GC (nt 6714 to 6736); and HXB2/G312A, 5′ ATC CAG AGA GCA CCA GGG AGA GCA (nt 6726 to 6749).

Cos-1 cells ($10^6$) were transfected with HIV-1 proviral vector DNA (20 μg) by using the calcium phosphate precipitation method as described elsewhere (11, 14). Twenty-four hours after transfection, the Cos-1 cells were cocultivated with $2 \times 10^6$ Sup-T1 cells, a T-cell line known to express high levels of surface CD4, making it well suited for HIV-1 infection (36). On day 3 postinfection, the cultures were transferred to T-75 flasks and maintained in culture with periodic feeding. Virus infection was monitored daily by measuring reverse transcriptase (RT) in a microtiter assay (45) and by PCR amplification and sequencing of the V3 region of the viral envelope (14). As can be seen in Fig. 1A, RT activity from the wild-type culture (HXB2/10) showed peak levels at day 4 postinfection. This peak of activity coincided with the observation of syncytia in infected cells. In comparison, the 10/G312T mutant culture failed to produce any measurable RT activity until day 25 postinfection. A similar pattern of delayed infectivity for the 10/G312T mutant was found in four independent experiments. Lower levels of peak RT activity were consistently observed for the mutant culture, which may reflect an impairment in the transmission of virus containing the G312T mutation.

Viral infectivity of the 10/G312T mutant was also tested by transfecting proviral vector DNA directly into Sup-T1 or AA5 cells by using DEAE-dextran (46). The AA5 cell line is an Epstein-Barr virus-transformed B-cell line that expresses high levels of CD4 and is exceptionally permissive for HIV-1 infection (3). The following day, the transfected cells were transferred to T-75 flasks and were maintained in culture with periodic feeding while infection was monitored as described above. Although the actual rate of infection with this direct transfection procedure was somewhat slower than that seen with the indirect cocultivation protocol (Fig. 1B compared with Fig. 1A), the same relative pattern was observed. Specifically, the production of mutant virus was delayed until days 38 to 42 postinfection, while the wild-type construct yielded a peak of viral activity by day 8 postinfection. The mutant culture demonstrated approximately 3.5-fold-higher RT levels at peak activity relative to the wild-type culture, possibly due to reduced cell killing by the mutant. Syncytium formation with an envelope protein containing a G312T mutation has previously been shown to be defective (14).

The G312T mutation was designed to incorporate a three-

TABLE 1. Endpoint titration analysis of rA312 and rR306 revertant, 10/G312A cloned, and HXB2/10 wild-type viruses in Sup-T1 and AA5 cells

| Virus | $TCID_{50}$[a] | |
|---|---|---|
| | Sup-T1 | AA5 |
| HXB2/10 | 14,465 | 231,442 |
| rA312 | 14,465 | <40 |
| 10/G312A | 28,858 | <40 |
| rR306 | 57,860 | >57,860 |

[a] $TCID_{50}$ values were calculated by the method of Reed and Muench (29) and represent the highest virus dilution which infected 50% of cells. Results are representative of three separate experiments.

base-pair change, from GGA to ACT, in codon 312 of the envelope gene in order to minimize the possibility of simple reversion in culture. The stability of this mutation was subsequently determined for progeny from each of the transfection experiments described above. A 660-bp region containing the V3 loop was amplified by PCR at various time points during the course of infection and used directly for sequencing. The DNA amplified from the wild-type cultures was found to be unaltered throughout infection. DNA amplified at early time points (day 8) from the mutant culture following direct transfection (Fig. 1B) was found to maintain the engineered ACT threonine codon at position 312. The ACT sequence at codon 312 was also identified in viral DNA up to day 35 posttransfection. However, by day 40 and coincident with the peak of RT activity, a nucleotide change that resulted in an alanine substitution (GCT) at position 312 was found in the first position of this codon. Sequencing of the entire envelope gene from the mutant culture revealed this to be the only change. Moreover, this same codon change was also identified in DNA isolated from mutant cultures following cocultivation (Fig. 1A). The consistency with which this particular substitution was generated indicates strong selective pressure imposed by the defective nature of the 10/G312T virus. The resulting revertant virus is referred to as rA312. Interestingly, an alanine residue in the first position of the tetrameric tip of the V3 loop (AGPR) has also been identified in natural HIV-1 isolates, albeit at low frequency (19).

Cell-free virus particles derived from Cos-1 cells transfected with the G312T proviral DNA were found to be noninfectious on Sup-T1 cells. No significant RT activity was detectable in culture supernatants monitored up to 60 days (data not shown). Sato et al. (31) have shown that HIV-1-specific cell-to-cell fusion can occur within an hour of cocultivation, in contrast to cell-free virus infection, which may take days. Our results would be consistent with the initial virus spread being mediated by cell-to-cell transmission, with RT levels remaining low until selection of the revertant generates a productive infection. Moreover, the G312T envelope protein has been previously found to be defective in syncytium formation in the HeLa-T4 assay (14).

The relative infectious titers of the rA312 revertant and wild-type viruses were determined by endpoint dilution titration assays in Sup-T1 and AA5 cells (15). The 50% tissue culture infectious dose ($TCID_{50}$) for each virus stock was calculated by the method of Reed and Muench (29). As outlined in Table 1, the infectious titer of the wild-type virus (HXB2/10) was a log unit higher on the AA5 cells compared with the Sup-T1 cells. The enhanced infectivity of this virus on AA5 cells has been previously reported (15). The rA312 revertant virus was as infectious as the wild-type virus on

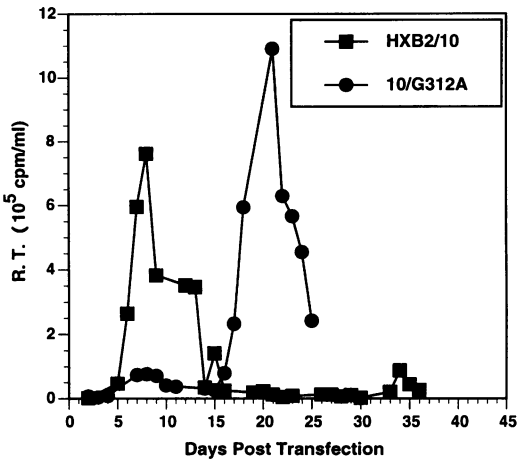


FIG. 2. Infectivity profiles of 10/G312A envelope mutant and HXB2/10 wild-type viruses generated by transfection of proviral DNA directly into AA5 cells. RT levels in culture supernatants were measured daily for 36 days posttransfection.

Sup-T1 cells but exhibited a reduced ability to infect AA5 cells, with a $TCID_{50}$ of less than 40. To determine if the A312 substitution was responsible for the altered phenotype on AA5 cells, an independent molecular clone, 10/G312A, was constructed in the chimeric proviral clone by using the two-step oligonucleotide-directed mutagenesis method described above. Virus was produced by cocultivation of transfected Cos-1 cells with Sup-T1 cells, and the G312A mutation was confirmed by sequence analysis of PCR-amplified DNA from the peak of virus production, which occurred 8 days postinfection (data not shown). In titration studies on Sup-T1 and AA5 cell lines, the 10/G312A cloned virus was found to have a pattern of infectivity identical to that of the revertant virus on both cells types, with viral titer reduced by several log units on the AA5 cells (Table 1). The data clearly demonstrate that a single amino acid change in the V3 loop is sufficient to alter the tropism of the virus on AA5 cells.

To address whether the tropism differences were dependent on virus entry, the initial entry steps were bypassed by transfecting the 10/G312A proviral DNA directly into Sup-T1 or AA5 cells. As expected, no alteration of the V3 loop sequence was detected in virus produced in Sup-T1 cells (data not shown). In the AA5 direct transfection experiment, shown in Fig. 2, a peak of RT activity from the mutant culture was unexpectedly observed approximately 14 days later than with the wild-type virus. DNA sequence analysis of the V3 loop region from this mutant culture revealed a second-site change in addition to the G312A mutation which was still present. The second change was identified at position 306, whereby the serine codon (AGT) was changed to CGT, encoding an arginine residue. This revertant virus is designated rR306. When titrated on Sup-T1 and AA5 cells, rR306 demonstrated a productive infection in both cell types (Table 1). Relative to the parental cloned virus (10/G312A) on AA5 cells, the titer of the revertant rR306 virus on this cell line was observed to be enhanced by several log units (Table 1). We have, in addition, generated a double mutant which encodes both the S306R and G312A substitutions within the BH10 envelope background. This cloned virus, 10/S306R,G312A, is also infectious on AA5 cells (Table 2). Thus, it appears that the occurrence of this second-site change within the V3 loop is sufficient to alter the virus phenotype on the AA5 cell line.

TABLE 2. Effect of envelope background on the infectivity of the G312A cloned mutant viruses in Sup-T1 and AA5 cells

| Virus | $TCID_{50}$[a] | |
|---|---|---|
| | Sup-T1 | AA5 |
| HXB2 | 5107 | 20,431 |
| HXB2/G312A | 319 | 5207 |
| HXB2/10 | 14,465 | 231,442 |
| 10/G312A | 28,858 | <40 |
| 10/S306R,G312A | 7177 | 14,465 |

[a] $TCID_{50}$ values were calculated by the method of Reed and Muench (29) and represent the highest virus dilution which infected 50% of cells. Results are representative of three separate experiments.

The V3 loop mutations described above were engineered into the chimeric HIV-1 vector (HXB2/10), in which the *Sal*I-*Bam*HI envelope region of the HXB2 genome was replaced with the corresponding region from the BH10 strain. Of the eight amino acid differences within the gp120 from HXB2 and BH10, only one occurs within the V3 loop at residue 306, which is serine in BH10 and arginine in HXB2. Incorporation of an arginine residue at position 306 in the rR306 revertant converted the sequence of the BH10 envelope to one which resembles the HXB2 envelope protein sequence. The R306 codon (CGT) in this revertant virus is, however, unique and does not match the R306 codon in our HXB2 clone, which is AGA. To assess the importance of the whole envelope moiety and the affinity of the virus for a particular cell type, we constructed clone HXB2/G312A, in which the glycine at residue 312 in the V3 loop of gp120 from HXB2 was replaced with alanine by using the two-step oligonucleotide mutagenesis method described above. Virus was produced as before by transfection into Cos-1 cells followed by Sup-T1 cocultivation. In endpoint titrations, the HXB2/G312A virus was found to be infectious on both Sup-T1 and AA5 cells (Table 2). Enhanced infectivity of HXB2/G312A in the AA5 line compared with Sup-T1 cells was evident. This result is in contrast to the infectivity profiles observed with the viruses containing the G312A mutation within the BH10-specific envelope background (i.e., 10/G312A and rA312). Therefore, the background envelope sequence of a virus containing a G312A mutation is important, particularly for infectivity on AA5 cells.

Interestingly, the amino acid at codon 306 position has been reported to have a controlling influence on the tropism of other HIV-1 molecular clones (6, 8). deJong et al. (7) have demonstrated that the presence of a serine-to-arginine change at codon 306, along with several nonsilent mutations in the right side of the V3 loop, was responsible for altering the virus phenotype on different lymphocyte cell lines. The syncytium-inducing ability of these viruses was also affected by these mutations. Revertants in their system which encoded second-site changes in the GPGR tip sequence were identified. In our system, the tip sequence was initially perturbed by introducing the G312A mutation, and we selectively identified revertants containing an S306R change. These results suggest that there is a degree of interplay among the V3 loop residues which helps to control its function. Other reports (7, 8, 37) have shown that the conformation and overall charge of the V3 loop can also influence the biological function of the gp120 protein. In addition, others have observed functional interactions between amino acid residues both within the V3 loop as well as outside, within the C2 domain (37, 44). In particular, mutations within these regions were shown to compensate each other and partially restore the function of gp120. The crystal structure of the V3 loop has recently been elucidated (9), and Ghiara et al. demonstrated a strong structural requirement for a glycine at position 312 (referred to as $Gly^{P319}$) which helps to maintain the S-shaped conformation of the loop.

Analysis of V3 loop sequences derived from cloned and primary HIV-1 isolates has revealed a distinct pattern of amino acid substitutions within this heterogeneous region that have a direct correlation with virus phenotype (24). A combination of nonconservative basic substitutions involving positions 11, 24, and/or 32 in the V3 loop, plus the loss of an acidic residue at position 25, appear to be the controlling features. Our results would be supportive of this correlation. Positions 11 and 25 in the V3 loop correspond to residues 306 and 322, respectively, of the envelope protein in our HIV-1 clones. While residue 322 is invariant in our clones, encoding for a lysine, it is only in combination with a basic residue at 306 that we observed a switch in the ability to infect AA5 cells, for example, with the revertant virus, rR306, and the cloned virus, HXB2/G312A. For the HXB2/G312A clone, the G312A mutation was introduced into the V3 loop of the molecular clone HXB2, which naturally has arginine at position 306. The amino acid sequence of the V3 loop region is identical for the HXB2/G312A, 10/S306R,G312A, and rR306 viruses. All are able to support replication in the AA5 cell line. However, preliminary experiments with the cloned viruses HXB2/G312A and 10/S306R,G312A and the revertant virus rR306 indicated that syncytium formation in AA5 cells infected with either rR306 or 10/S306R,G312A was greatly diminished compared with HXB2/G312A (data not shown). Analysis of the envelope proteins from the 10/S306R,G312A and rR306 viruses in the HeLa-T4 syncytium assay (14) might possibly provide more direct evidence for their impaired function. These assays are currently in progress in another laboratory. From these observations, it appears that the S306R modification is necessary for infectivity on AA5 cells; however, it is not sufficient to fully alter the phenotype in terms of syncytium formation. These data also suggest that the tropism differences observed with our various viruses containing the G312A alteration may be more complex than a simple switch from an NSI to an SI phenotype as was evident in cloned viruses in a study by deJong et al. (7). Recent reports have implicated roles for both the V1 and V2 regions in cellular tropism and syncytium induction (1, 12); however, we have discovered no sequence differences in the V1 and V2 regions of our mutant viruses (data not shown). Further studies will be necessary to elucidate the potential interactive role for other hypervariable domains in this complex process.

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