

Single Amino Acid Substitution in Constant Region 1 or 4 of gp120 Causes the Phenotype of a Human Immunodeficiency Virus Type 1 Variant with Mutations in Hypervariable Regions 1 and 2 To Revert

WEI-KUNG WANG, MAX ESSEX, AND TUN-HOU LEE*

Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts 02115

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The second major cysteine loop of human immunodeficiency virus type 1 envelope glycoprotein gp120 contains 5 to 11 consensus N-linked glycosylation sites, which is disproportionately higher than the number of such sites found in other regions of gp120. Amino acid substitutions introduced at three of six N-linked glycosylation sites in this region of an infectious molecular clone, HXB2, resulted in severe impairment of virus infectivity. Isolation and genetic characterization of a revertant of this mutant revealed an isoleucine-for-valine substitution at position 84 in constant region 1 and an isoleucine-for-methionine substitution at position 434 in constant region 4. Further mutational analysis indicated that either isoleucine substitution was sufficient to confer the revertant phenotype. These findings demonstrate that V1/V2 not only functionally interacts with C4, as previously reported, but also interacts with C1. The observation that compensatory changes do not involve regeneration of N-linked glycosylation sites in the second major cysteine loop suggests that replication of human immunodeficiency virus type 1 in vitro is independent of the presence of a disproportionate number of N-linked glycosylation sites within this loop.

Human immunodeficiency virus type 1 (HIV-1) isolates are genomically heterogeneous, particularly in the region of the envelope gene that encodes envelope glycoprotein gp120 (5, 12, 26, 28, 30, 41). Variation in gp120 is clustered in five discrete hypervariable regions, V1 to V5, which are interspersed between five constant regions, C1 to C5 (22, 26). Mature gp120 contains 18 highly conserved cysteine residues, which form nine disulfide bonds (18, 26). Leonard et al. determined the location of the nine disulfide bonds in a prototypic gp120 protein and proposed a secondary structure that groups gp120 residues into five major cysteine loops (18).

The second major cysteine loop has a complex double-loop structure with disulfide bonds involving cysteine residue pairs 119 and 205, 126 and 196, and 131 and 157 (28) (Fig. 1). This loop accounts for one-fifth of the gp120 coding sequence and includes hypervariable regions V1 and V2. Residues within this loop play important roles in cellular tropism (7, 13, 14, 31, 38), syncytium formation (2, 11, 34), and virus infectivity (34, 35).

Several reports have described functional interactions between the second major cysteine loop and other regions of gp120. An asparagine-for-serine substitution at position 128 in the V1 region restored the infectivity of a mutant with a glutamine-for-asparagine substitution at position 267 (equivalent to position 262 of the infectious molecular clone HXB2 [28]) in the C2 region (39, 40). In another study, a histidine substituted for a tyrosine at position 435 in the C4 region abrogated the recognition of gp120 by two conformation-dependent anti-V1/V2 monoclonal antibodies (20). Similarly, amino acid changes in C3 and C4 inhibited or enhanced the binding of several anti-V2 monoclonal antibodies to gp120 (23). Furthermore, a methionine-for-isoleucine substitution at position 418 (position 420 of HXB2) in the C4 region was responsible for

the revertant phenotype of a chimeric virus, pNL4-3CH14 (10). Thus far, no interaction between V1/V2 and constant region C1 or C5 has been reported.

The second major cysteine loop of gp120 contains 5 to 11 consensus N-linked glycosylation sites (Asn-Xaa-Ser/Thr), which is disproportionately higher than the number of such sites found in most other regions of gp120 (26). Although some consensus N-linked glycosylation sites are linked to insertions or deletions in V1 and V2, a relatively high number are conserved by isolates of different genetic clades (26). On the basis of analyses conducted with HXB2 (9), we previously reported that five N-linked glycosylation sites important for virus infectivity are clustered in the amino-terminal half of gp120 (17). Among the five, two are located in the second major cysteine loop (17). In this study, a variant with mutations simultaneously introduced to three N-linked glycosylation sites in the second major cysteine loop was constructed by site-directed mutagenesis. This mutant had severely impaired virus infectivity. A revertant virus was subsequently isolated and genetically characterized to determine if a disproportionately high number of N-linked glycosylation sites in the second major cysteine loop is required for HIV-1 replication in vitro and to characterize the interaction between V1/V2 and other regions of gp120.

HIV-1 variant with N-linked glycosylation site mutations in V1/V2 (dgN3). Within the second major cysteine loop of the infectious molecular clone HXB2, there are six N-linked glycosylation sites (9) (Fig. 1). By site-directed mutagenesis, three N-linked glycosylation sites at positions 136, 156, and 186 were simultaneously replaced with glutamine (16, 17). These sites were selected because mutations previously individually introduced at these sites showed no discernible effect on virus infectivity (17). To study the virus infectivity of this mutant, designated dgN3, 4 μ g of mutant DNA or wild-type DNA was transfected into 3×10^6 to 5×10^6 COS-7 cells, using a previously described DEAE-dextran method (15, 43). Cell-free

* Corresponding author. Mailing address: Department of Cancer Biology, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. Phone: (617) 432-1332. Fax: (617) 739-8348.

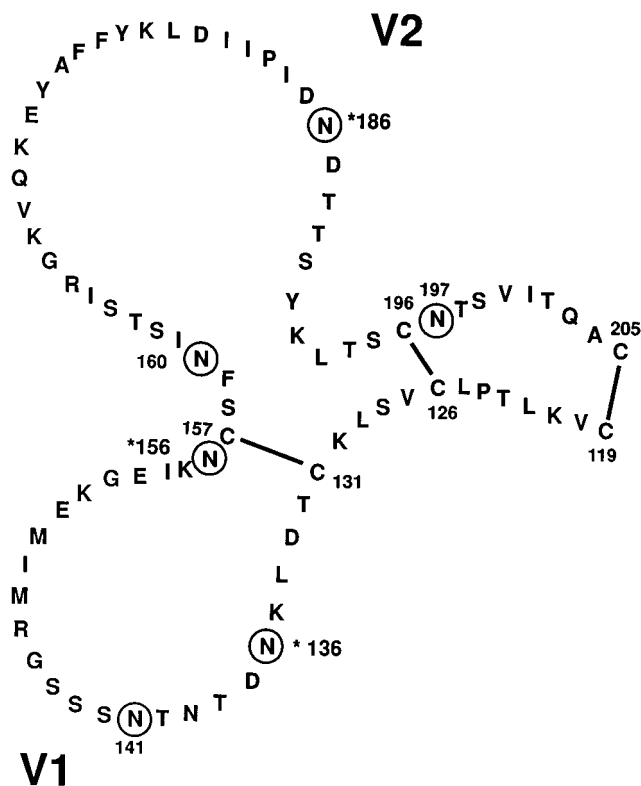


FIG. 1. Schematic drawing of the second major cysteine loop of gp120. This loop is characterized by two disulfide bonds involving cysteine pairs 119 and 205, and 126 and 196, near the base of the loop, and a third disulfide bond involving cysteine residues 131 and 157 (26). The six N-linked glycosylation sites within this loop are indicated by circles. Asterisks, N-linked glycosylation sites studied.

virus from the culture supernatant of COS-7 transfectants was collected 72 h posttransfection. Equal amounts of mutant and wild-type virus, as measured by reverse transcriptase (RT) activity, were used to infect CD4⁺ SupT1 cells and the cultures were monitored every 4 days for RT activity as described previously (17). As shown in Fig. 2A, no RT activity was detected in the dgN3-infected culture during the 32 days of follow-up.

Phenotypic revertant of dgN3 (dgN3R). The dgN3-infected cultures were continuously screened for the emergence of revertants that regain virus infectivity. Such revertants should, in theory, shed new light on which regions of gp120 functionally interact with V1/V2 and on whether a relatively large number of N-linked glycosylation sites in V1/V2 is required for virus infectivity. In one of three long-term cultures infected by dgN3, a high level of RT activity was detected at day 115, suggesting the emergence of revertant virus (data not shown). Cell-free supernatant from this culture was used to inoculate fresh SupT1 cells. A peak level of RT was detected around day 20 (Fig. 2B). This confirmed that revertant virus, designated dgN3R, emerged from the culture infected by dgN3 mutant. Western blot (immunoblot) analysis of cell lysates derived from the dgN3R-infected culture revealed a mutant gp120 with a migration rate slightly higher than that of wild-type gp120 (data not shown). This suggests that the revertant virus contained a gp120 distinct from that of the wild-type virus.

Infectious molecular clone containing dgN3R envelope sequence. To further characterize the revertant virus, a chimeric clone of HXB2, designated dgN3R.4, which contains 470 C-terminal residues of gp120 and 100 N-terminal residues of gp41 of dgN3R, was constructed. Briefly, 5×10^6 SupT1 cells

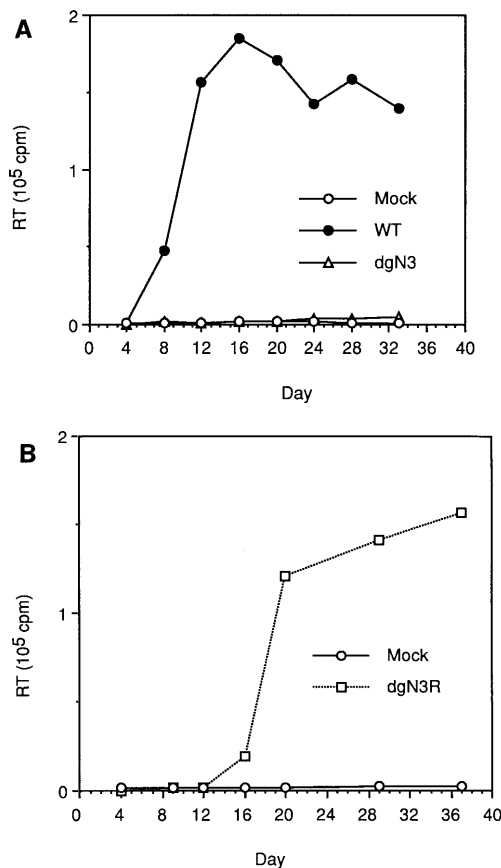


FIG. 2. (A) Growth kinetics of wild-type (WT) virus and mutant virus dgN3. (B) Growth kinetics of the revertant virus dgN3R in CD4-positive SupT1 cells. RT activity in culture supernatants was monitored for 32 days. "Mock" refers to cultures infected by cell-free supernatants of mock-transfected COS-7 cultures.

of the revertant-infected culture were pelleted and high-molecular-weight DNA was purified by a standard proteinase K digestion-phenol extraction technique (19). Two micrograms of DNA was subjected to 30 cycles of PCR amplification as described previously (36). Oligonucleotide primers $-7A$, 5'-GAAAGAGCAGAAGACAGTGG-3' (corresponding to positions 6202 to 6221 of the HXB2 genome [28]), and 619B, 5'-CAGAGATTTATTACTCCAAC-3' (positions 8061 to 8080), were designed to amplify a 1.87-kb fragment of the envelope gene. This fragment contains the entire gp120 protein and 100 N-terminal residues of gp41. Amplified product was cloned to shuttle vector PCR II (Invitrogen, San Diego, Calif.) as described previously (36). DNA obtained from colonies with inserts were digested with *Kpn*I and *Bsm*I. The 1.7-kb *Kpn*I-*Bsm*I fragment, which contains 470 C-terminal residues of gp120 and 100 N-terminal residues of gp41, was cloned to shuttle vector EB-PGEM 7, which contains the 2.7-kb *Eco*RI-*Bam*HI fragment of HXB2. The 2.6-kb *Sal*I-*Bam*HI fragment containing the *Kpn*I-*Bsm*I insert was cut to replace the 2.6-kb *Sal*I-*Bam*HI fragment of HXB2. The envelope-chimeric clones that were generated were screened for virus infectivity.

Cell-free virus from the supernatants of COS-7 cells transfected with dgN3R.4 and wild-type virus, as well as dgN3R from the original culture, was collected for subsequent study of virus infectivity. Equal amounts of virus, normalized by RT activity, were used to infect SupT1 cells. As shown in Fig. 3, the

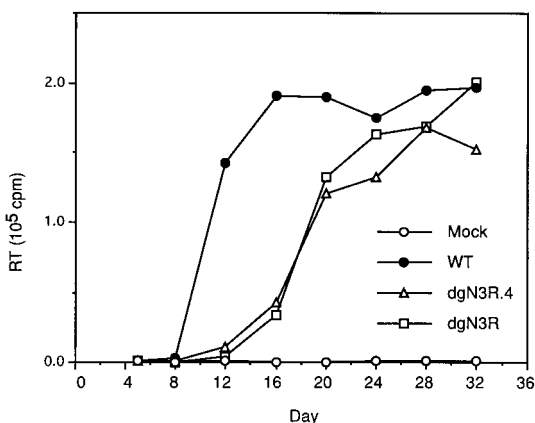


FIG. 3. Growth kinetics of wild-type (WT) virus, revertant virus dgN3R, and the molecular clone of revertant virus dgN3R.4 in CD4-positive SupT1 cells. RT activity in culture supernatants was monitored for 32 days. "Mock" refers to cultures infected by cell-free supernatants of mock-transfected COS-7 cultures.

growth kinetics of dgN3R.4 and dgN3R were comparable, although both lagged somewhat behind the wild-type virus. This indicates that the 1.7-kb *KpnI-BsmI* envelope fragment amplified from the dgN3R virus is sufficient to confer the revertant phenotype.

Compensatory amino acid changes in dgN3R. The 1.7-kb fragment of dgN3R.4 was completely sequenced to identify amino acid changes that could account for the revertant phenotype (29). The original N-linked glycosylation site mutations were retained at positions 136, 156, and 186 (Table 1). No new N-linked glycosylation sites were generated in the second major cysteine loop. However, two additional amino acid substitutions involving highly conserved residues in C1 and C4 were found. One was located at position 84 in the C1 region and involved substitution of isoleucine (ATA) for valine (GTA). The other was located at position 434 in the C4 region and involved substitution of an isoleucine (ATA) for methionine (ATG) (Table 1). These findings suggest the possibility of a functional interaction between the second major cysteine loop and C1 and/or C4.

Single substitution in C1 or C4 confers the revertant phenotype. To determine whether the isoleucine-for-valine substitution at position 84 or the isoleucine-for-methionine substitution at position 434 can independently restore the infectivity of dgN3, three additional mutant molecular clones were constructed by site-directed mutagenesis with the 2.7-kb *EcoRI-BamHI* fragment of dgN3 as a template (16, 17). These molecular clones, designated 84VI/dgN3, 434MI/dgN3, and 84VI+434MI/dgN3, all contain the three original N-linked glycosylation site mutations in the second major cysteine loop. In addition, the 84VI/dgN3 clone contains an isoleucine-for-valine substitution at position 84, the 434MI/dgN3 clone has an isoleucine-for-methionine substitution at position 434, and the

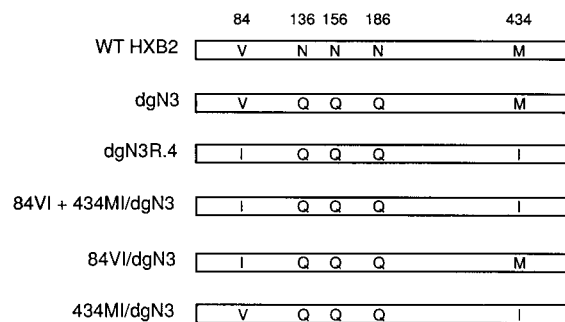


FIG. 4. Schematic drawing of mutant and revertant constructs. Amino acids at various positions are shown. WT, wild type.

84VI+434MI/dgN3 clone has both substitutions at positions 84 and 434 (Fig. 4). The mutant envelope proteins of 84VI/dgN3, 434MI/dgN3, and 84VI+434MI/dgN3 all migrated faster than that of the wild-type virus (data not shown). This is consistent with the results of our genetic analyses, which indicated that phenotypic reversion of the dgN3 mutant did not involve the generation of new N-linked glycosylation sites within or outside the second major cysteine loop. As shown in Fig. 5, the growth kinetics of viruses derived from 84VI/dgN3 or 434MI/dgN3 transfectants were comparable to those of 84VI+434MI/dgN3. This suggests that single amino acid substitutions at either position 84 in C1 or 434 in C4 are sufficient to compensate for the impaired virus infectivity of mutant dgN3.

Single amino acid substitutions in C1 (isoleucine for valine at position 84) or C4 (isoleucine for methionine at position 434) were able to restore the infectivity of a variant with three N-linked glycosylation site mutations within the second major cysteine loop. This suggests a functional interaction between V1/V2 and C1, as well as between V1/V2 and C4. Functional interactions between different regions of gp120 have been reported (10, 20, 23–25, 33, 39, 40, 42). A schematic drawing summarizing these interactions is shown in Fig. 6. Since V1 has been reported to interact with C2, and C2 has been reported to interact with C1, our finding that V1/V2 interacts with C1 is compatible with these observations (25, 39, 40). In addition, our finding that V1/V2 interacts with C4 is consistent with previous reports that V1 interacts with C2 (39, 40), C2 interacts with V3 (33, 39), and V3 interacts with C4 (24, 42). Our finding also confirms previous reports of a functional interaction between V1/V2 and C4 (10, 20, 23). To our knowledge, the only region of gp120 that has not been structurally or functionally linked to other parts of gp120 is V5.

The observation that compensatory changes do not involve the regeneration of N-linked glycosylation sites suggests that the N-linked glycosylation sites at positions 136, 156, and 186 in the second major cysteine loop are dispensable for virus infectivity in SupT1 cells in vitro. Similar results were found with peripheral blood mononuclear cells. The infectivity of

TABLE 1. Compensatory amino acid changes in revertant dgN3R.4

Construct	Amino acid residue (codon) at position ^a :				
	84	136	156	186	434
HXB2 (wild type)	V (GTA)	N (AAT)	N (AAC)	N (AAT)	M (ATG)
dgN3	V (GTA)	Q (CAG)	Q (CAG)	Q (CAG)	M (ATG)
dgN3R.4	I (ATA)	Q (CAG)	Q (CAG)	Q (CAG)	I (ATA)

^a Numbers according to HXB2 sequence (28).

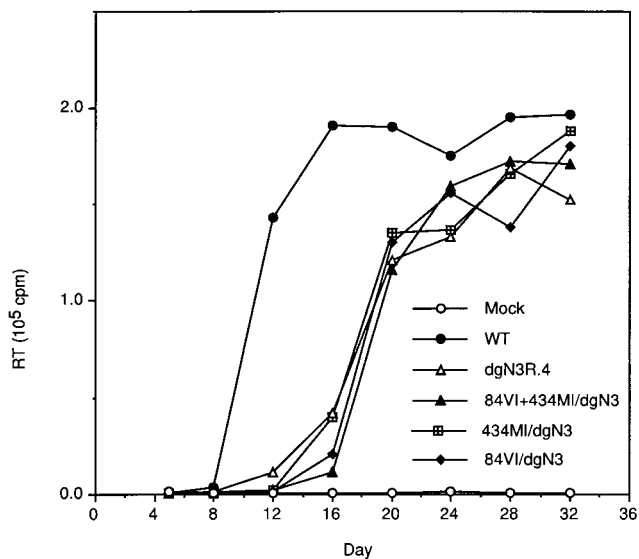


FIG. 5. Growth kinetics of wild-type (WT) virus and the revertant viruses dgN3R.4, 84VI+434MI/dgN3, 84VI/dgN3, and 434MI/dgN3 in CD4-positive SupT1 cells. RT activity in culture supernatants was monitored for 32 days. "Mock" refers to cultures infected by cell-free supernatants of mock-transfected COS-7 cultures.

dgN3 was severely impaired, but it could be restored by revertants dgN3R.4, 84VI/dgN3, 434MI/dgN3, and 84VI+434MI/dgN3 (data not shown). The loss of infectivity of mutant dgN3 may be due to the combined effects of the three glutamines substituted for the three asparagines at these positions, although individual mutations at these positions do not affect virus infectivity (17). Alternatively, the removal of three N-linked glycosylation sites may alter N-linked glycosylation, thus rendering dgN3 noninfectious.

Our finding that three N-linked glycosylation sites within the second major cysteine loop of HIV-1 gp120 are dispensable for virus infectivity *in vitro* suggests that the conservation of a relatively high number of N-linked glycosylation sites within this loop *in vivo* is probably unrelated to virus replication. Several studies have suggested that carbohydrate moieties in the hypervariable regions may modulate the antigenic properties of gp120 and may contribute to the virus's ability to evade the immune system (1, 3, 4, 6, 8, 37). Similarly, analyses of sequential samples from HIV-1-infected individuals have revealed changes in N-linked glycosylation sites in variable regions *in vivo* (21, 32). Viruses associated with progression to simian AIDS tend to gain potential N-linked or O-linked glycosylation sites in the V1 and V4 regions (27). These observations resonate with our finding that compensatory changes do

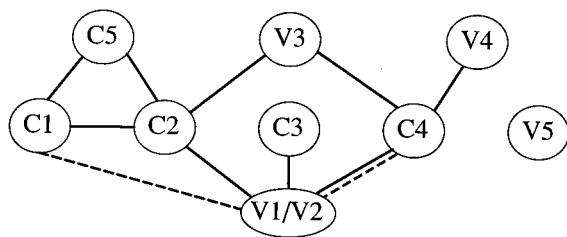


FIG. 6. Schematic drawing of functional interactions between different regions of gp120. Solid lines indicate previously reported interactions; dashed lines indicate the interactions reported here.

not involve regeneration of N-linked glycosylation sites. Conservation of a relatively high number of N-linked glycosylation sites in the second major cysteine loop of gp120 may be a strategy of the virus to escape immune surveillance.

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