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Minimal Requirements for the Human Immunodeficiency Virus Type 1 V3 Domain To Support the Syncytium-Inducing Phenotype: Analysis by Single Amino Acid Substitution

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The third variable domain (V3) of the human immunodeficiency virus type 1 external envelope contains determinants of cell tropism, cytopathicity, and infectivity and elicits antibodies able to block infectivity in vitro and in vivo. Our study encompassed point-mutational analysis of HXB-2 viruses containing patient-derived V3 regions and expressing a non-syncytium-inducing, low-replicating phenotype in T-cell line SupT1. The mutation within V3 of a serine at position 306 into an also naturally occurring arginine (S to R) required an additional, naturally occurring mutation at position 320 (aspartate to glutamine, D to Q) or 324 (aspartate to asparagine, D to N) for full expression of the syncytium-inducing, high-replicating (SI) phenotype. The naturally occurring mutation of an aspartate into an arginine at position 320 (D to R) was sufficient for production of the SI phenotype. This study proves that introduction of a positively charged amino acid at position 306 or 320, previously shown to be strongly associated with the SI phenotype in field isolates (R. A. M. Fouchier, M. Groenink, N. A. Kootstra, M. Tersmette, H. G. Huisman, F. Miedema, and H. Schuitemaker, J. Virol. 66:3183–3187, 1992), is minimally required for production of SI viruses. In addition, naturally occurring mutations at residue 324 also modulate the virus phenotype.

Non-syncytium-inducing, low-replicating (NSI) human immunodeficiency virus type 1 (HIV-1) isolates are predominant in the asymptomatic stage of HIV-1 infection (11). The emergence of syncytium-inducing, high-replicating (SI) viruses in the course of HIV-1 infection is correlated with subsequent rapid progression to AIDS (11, 12). The type-specific neutralizing domain of HIV-1 located in the V3 region of the envelope has been shown to be involved in determination of syncytium formation, host range, and infectivity of HIV-1 (1, 2, 4–6, 8, 10, 13), but other regions are involved as well (9, 13).

Using an HXB-2 background, we have developed a cassette system in which we can create viruses that differ in the V3 region only. Regions of the virus other than V3 that influence syncytium-forming capacity and replication rate (9, 13) are not affected. Insertion into this cassette of V3 sequences derived from NSI and SI isolates from the same patient, with mutations at a limited number of sites, resulted in hybrid viruses with marked differences in SI capacity corresponding to the phenotype of the original isolates (2, 7). Comparison of a large number of V3 sequences from field isolates revealed a strong correlation between positively charged amino acid residues at two of these sites and the SI phenotype (3). The aim of this study was to determine the minimal number of naturally occurring amino acid changes within the V3 region required for production of an SI phenotype starting from two naturally occurring V3 regions derived from NSI isolates from two patients in whom a switch to SI variants occurred.

HIV-1 isolates 168.1 (NSI) and 168.10 (SI) are sequential isolates obtained from the same asymptomatic individual by coculture of his peripheral blood lymphocytes (PBLs) with donor PBLs (2, 11). In T-cell line SupT1, the syncytium-inducing capacity of chimeric HXB-2 virus containing only the V3 region from either 168.1 or 168.10 accorded with the phenotype of HIV-1 isolates 168.1 (NSI) and 168.10 (SI), respectively (2). The V3 region of the 168.1 NSI isolate differed by four amino acids from the V3 region of the 168.10 SI isolate. A set of 16 chimeric viruses covering all possible combinations of the four amino acid changes (Fig. 1A, 2A, and 3A) was constructed. The complete set of molecular clones was transfected in SupT1 cells; syncytium formation, cell numbers (Fig. 1C, 2C, and 3C), and p24 core antigen production were monitored for 14 days after transfection (Fig. 1B, 2B, and 3B). Molecular clones containing a single amino acid of the 168.10 V3 sequence incorporated in the 168.1 sequence were still unable to induce syncytia, except for the 168.1/R variant (S-to-R change at position 306) (Fig. 1). Transfection of the 168.1/R molecular clone in SupT1 cells did result in syncytium formation. The number of syncytia, however, was consistently lower than that observed for the 168.10 clone. Also, in contrast to 168.10, transfection with 168.1/R did not result in complete cell death in the culture. Molecular clones containing a combination of amino acids of the 168.10 sequence at positions 317 (A to T), 320 (D to Q), and 324 (D to N) maintained the 168.1 phenotype, except for the 168.1/QN and 168.1/TQN variants (Fig. 2). The 168.1/QN and 168.1/TQN molecular clones produced viruses that induced a low number of syncytia upon prolonged culture and that showed a slightly elevated replication rate compared with the 168.1 chimeric virus. Introduction within the 168.1 V3 sequence of the R (position 306) of the 168.10 V3 sequence, in combination with the Q (position 320) and/or N (position 324) mutation of the 168.10 V3 sequence (168.1/RQ, 168.1/RN, and 168.1/RQN vari-

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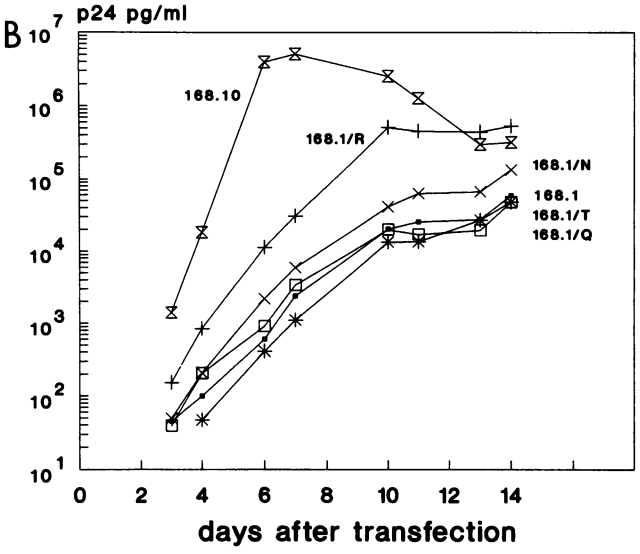
A

molecular clone	V3 amino acid sequence (296 306 317 320 324)	charge*	SI phenotype**
168.1	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+	-
168.10	----------R----------T--Q---N------	6+	+
Mutagenesis set			
168.1/R	----------R----------	4+	± ***
168.1/T	----------T----------	3+	-
168.1/Q	----------Q----------	4+	-
168.1/N	----------N------	4+	-

B



C

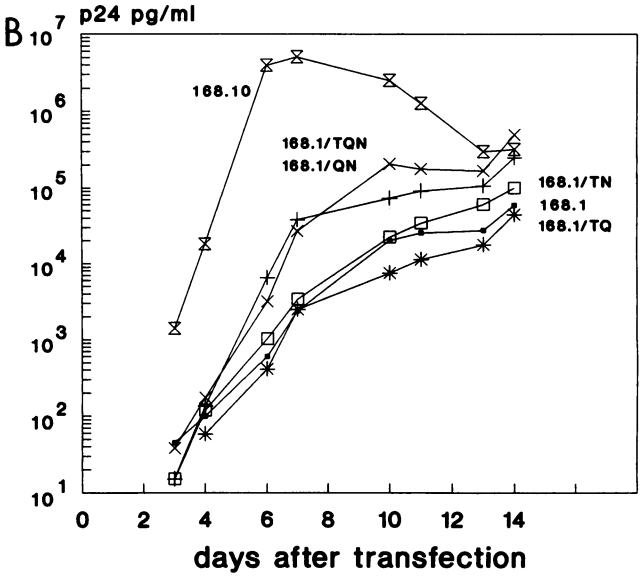
molecular clone	syncytium formation: 3*	4	6	7	10	11	13	14
168.1	-	-	-	-	-	-	-	-
168.10	+	+±	++++	++++/d	d	d	d	d
Mutagenesis set								
168.1/R	-	-	+	+±	++±	++	+	+
168.1/T	-	-	-	-	-	-	-	-
168.1/Q	-	-	-	-	-	-	-	±
168.1/N	-	-	-	-	-	-	-	-

FIG. 1. Characteristics of chimeric molecular clones containing single amino acid mutations. (A) Name of molecular clone, sequence and overall charge of V3 region at pH 7.0 (*), and phenotype of the corresponding chimeric virus in SupT1 cells (**). ***, characteristics of this phenotype are explained in the text. The method for construction of the chimeric viruses and the polymerase chain reaction-based mutagenesis of the V3 regions have been described in detail before (2). In brief, the V3 region between the two cysteines was amplified by polymerase chain reaction. The amplified V3 region was inserted in a plasmid containing the *Nco*I-to-*Bam*HI fragment of HXB-2. Subsequently, this chimeric V3 envelope fragment was cloned into a full-length molecular HXB-2 clone lacking this *Nco*I-to-*Bam*HI fragment. Thus, a complete molecular HXB-2 clone chimeric for the V3 region was created. (B) Viral replication of the chimeric molecular clones shown in panel A. The molecular clones shown in Fig. 1 to 3 were transfected at the same time with the same batch of SupT1 cells, allowing direct comparison of the replication rates of all of these chimeric viruses. The experiment was performed three times; representative results of one are shown. The method used to monitor replication and syncytium formation has been described before (2). In brief, 1 μg of the molecular clones was transfected by electroporation into 5×10^6 SupT1 cells. All cultures were split at days 4, 7, and 11 by adding 4 ml of medium to 2 ml of culture. To monitor viral replication, p24 core antigen was measured by using a capture enzyme-linked immunosorbent assay. Direct sequencing of the V3 region of DNA from the transfected cells at 14 days postinfection revealed that no changes in the V3 region had occurred. (C) Syncytium induction of the molecular clones shown in panel A. −, no syncytia present; +, some syncytia present; ++, syncytia in every 10 by 10 field; +++, high number of syncytia, but still normal cells present; ++++, only syncytia present; +±, number of syncytia intermediate between + and ++; d, all cells dead, no syncytia present. Syncytia were scored by two workers independently. *, days after transfection.

A

molecular clone	V3 amino acid sequence (296 306 317 320 324)	charge*	SI phenotype**
168.1	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+	-
168.10	----------R----------T--Q---N------	6+	+
Mutagenesis set			
168.1/TQ	----------T--Q----------	4+	-
168.1/TN	----------T------N------	4+	-
168.1/QN	----------Q---N------	5+	-
168.1/TQN	----------T--Q---N------	5+	-

B



C

molecular clone	syncytium formation: 3*	4	6	7	10	11	13	14
168.1	-	-	-	-	-	-	-	-
168.10	+	+±	++++	++++/d	d	d	d	d
Mutagenesis set								
168.1/TQ	-	-	-	-	-	-	-	±
168.1/TN	-	-	-	-	-	-	-	-
168.1/QN	-	-	±	±	+±	+	±	++
168.1/TQN	-	-	±	±	+±	+±	+±	++

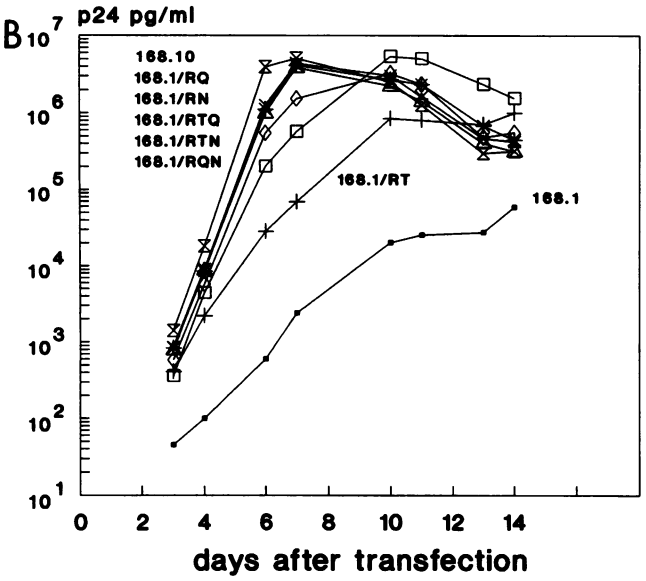
FIG. 2. Characteristics of chimeric molecular clones containing a combination of mutations at positions 317, 320, and 324. (A) Name of molecular clone, sequence and overall charge of V3 region at pH 7.0 (*), and phenotype of the corresponding chimeric virus in SupT1 cells (**). (B) Viral replication of the chimeric molecular clones shown in panel A. (C) Syncytium induction of the molecular clones shown in panel A. −, no syncytia present; +, some syncytia present; ++, syncytia in every 10 by 10 field; +++, high number of syncytia, but still normal cells present; ++++, only syncytia present; +±, number of syncytia intermediate between + and ++; d, all cells dead, no syncytia present. *, days after transfection.

ants), produced chimeric viruses with the 168.10 SI phenotype (Fig. 3). It appeared from the phenotype of the variants 168.1/R versus 168.1/RT, 168.1/QN versus 168.1/TQN, and 168.1/Q versus 168.1/TQ that the alanine-to-threonine mutation at position 317 did not influence the phenotype of the chimeric viruses.

Previously, we also analyzed the V3 regions of sequentially obtained isolates from patient 479 (7), which in PBLs also showed a switch from the non-syncytium-inducing to the syncytium-inducing phenotype. The V3 region of patient 479 is representative for an additional class of V3 regions with a positively charged amino acid at position 320 (3).

A

molecular clone	V3 amino acid sequence (296 306 317 320 324)	charge*	SI phenotype**
168.1	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+	-
168.10	----------R----------T--Q---N------	6+	+
Mutagenesis set			
168.1/RT	----------R----------T----------	4+	± ***
168.1/RQ	----------R----------Q----------	5+	+
168.1/RN	----------R----------N----------	5+	+
168.1/RTQ	----------R----------T--Q---------	5+	+
168.1/RTN	----------R----------T------N------	5+	+
168.1/RQN	----------R----------Q---N------	6+	+



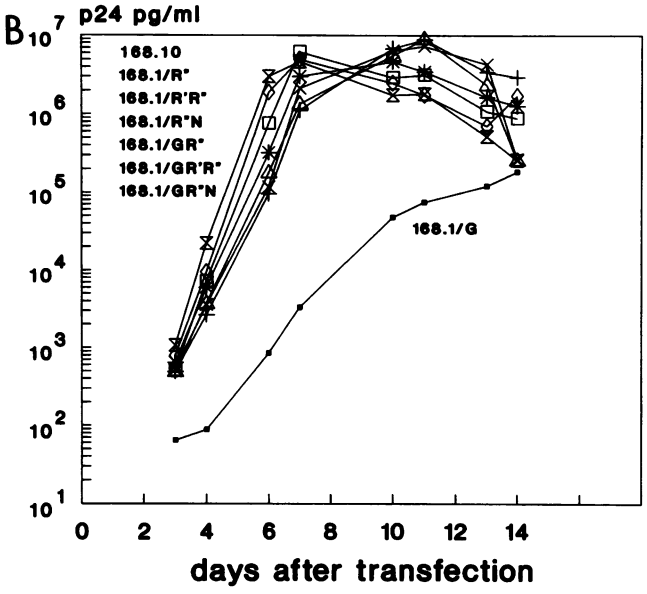
C

molecular clone	syncytium formation: 3*	4	6	7	10	11	13	14
168.1	-	-	-	-	-	-	-	-
168.10	+	+±	++++	++++/d	d	d	d	d
Mutagenesis set								
168.1/RT	±	±	++	++	++±	++±	+±	++
168.1/RQ	±	+	+++	+++±	d	d	d	d
168.1/RN	±	+	+++	+++	+++	+++	d	d
168.1/RTQ	±	+±	+++±	+++±	d	d	d	d
168.1/RTN	±	+±	+++	++++	++++	++++/d	d	d
168.1/RQN	±	+±	+++±	++++	d	d	d	d

FIG. 3. Characteristics of chimeric molecular clones containing the mutation at position 306 and a combination of mutations at positions 317, 320, and 324. (A) Name of molecular clone, sequence and overall charge of V3 region at pH 7.0 (*), and phenotype of the corresponding chimeric virus in SupT1 cells (**). ***, characteristics of this phenotype are explained in the text. (B) Viral replication of the chimeric molecular clones shown in panel A. (C) Syncytium induction of the molecular clones shown in panel A. −, no syncytia present; +, some syncytia present; ++, syncytia in every 10 by 10 field; +++, high number of syncytia, but still normal cells present; ++++, only syncytia present; +±, number of syncytia intermediate between + and ++; d, all cells dead, no syncytia present. *, days after transfection.

A

molecular clone	V3 amino acid sequence (296 306 314 320 324)	charge*	SI phenotype**
168.1	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+	-
168.10	----------R----------T--Q---N------	6+	+
479.4	----------G----------Q---N------	5+	-
479.6	----------G-------R----RR----------	8+	+
Mutagenesis set			
168.1/R*	----------R----------	5+	+
168.1/R*R*	----------RR----------	6+	+
168.1/R*N	----------R---N------	6+	+
168.1/G	----------G----------	3+	-
168.1/GR*	----------G----------R----------	5+	+
168.1/GR*R*	----------G----------RR----------	6+	+
168.1/GR*N	----------G----------R---N------	6+	+



C

molecular clone	syncytium formation: 3*	4	6	7	10	11	13	14
168.1	-	-	-	-	-	-	-	-
168.10	+	++	++++	++++/d	d	d	d	d
Mutagenesis set								
168.1/R*	-	+	++	++±	+++±	++++/d	±	++
168.1/R*R*	±	+	++±	++++	++++/d	d	d	d
168.1/R*N	±	+	+++	+++±	d	d	d	d
168.1/G	-	-	-	-	-	-	-	-
168.1/GR*	-	+	++	+++	+++±	++++/d	±	±
168.1/GR*R*	±	+	+++±	++++	d	d	d	d
168.1/GR*N	-	+	++	+++	++++/d	++++/d	±	±

FIG. 4. Characteristics of chimeric molecular clones containing 479-like mutations. (A) Name of molecular clone, sequence and overall charge of V3 region at pH 7.0 (*), and phenotype of the corresponding chimeric virus in SupT1 cells (**). (B) Viral replication of the chimeric molecular clones shown in panel A. (C) Syncytium induction of the molecular clones shown in panel A. −, no syncytia present; +, some syncytia present; ++, syncytia in every 10 by 10 field; +++, high number of syncytia, but still normal cells present; ++++, only syncytia present; +±, number of syncytia intermediate between + and ++; d, all cells dead, no syncytia present. *, days after transfection.

Again, in T-cell line SupT1, the syncytium-inducing capacity of the chimeric HXB-2 viruses containing only the V3 region from either 479.4 or 479.6 accorded with the phenotype of HIV-1 isolate 479.4 (NSI) or 479.6 (SI), respectively (7). The two V3 regions differed at four amino acid positions (Fig. 4A). In contrast to the isolates from patient 168, the switch from NSI to SI was accompanied by mutations at the right side of the V3 loop only. To investigate the role of the amino acid substitutions occurring naturally in the NSI and SI isolates from patient 479, seven additional 168.1-based molecular clones containing mutations at positions 306 (S to G), 320 (D to R), 319 and 320 (GD to RR), and 320 and 324 (D to

R and D to N) (Fig. 4A) were constructed. The mutation at position 306 (S to G) did not influence the phenotype of the chimeric virus. Molecular clones containing one or two mutations of 479.6 at the right side of the V3 loop produced viruses with the SI phenotype (Fig. 4). Conversion of an aspartate (D, negatively charged) into an arginine (R, positively charged) at position 320 within the 168.1 NSI V3 loop was sufficient to gain the SI phenotype.

In conclusion, naturally occurring mutations at positions 306, 320, and 324 of the V3 loop convert the 168.1 phenotype into the SI phenotype of 168.10. In variants that acquired the 168.10 SI phenotype, the overall charge of the V3 loop increased from +3 to +5. However, the position of the charge within the V3 loop also influenced the phenotype, as evidenced by differences in phenotype between 168.1/R and 168.1/Q or 168.1/N (overall charge, +4) and between 168.1/QN and 168.1/R", 168.1/RQ, or 168.1/RN (overall charge, +5). This study demonstrates that introduction of positively charged amino acids at position 306 or 320, previously shown to be strongly associated with the SI phenotype in field isolates (3), is minimally required for production of SI viruses. In addition, this study reveals that naturally occurring mutations at position 324 also modulate the virus phenotype.

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