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Phenotype-Associated Sequence Variation in the Third Variable Domain of the Human Immunodeficiency Virus Type 1 gp120 Molecule

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The third variable (V3) domain has been implicated in determining the human immunodeficiency virus (HIV) phenotype, including fusion capacity and monocytotropism. In a large set of primary HIV type 1 (HIV-1) isolates, V3 sequence analysis revealed that fast-replicating, syncytium-inducing isolates contained V3 sequences with a significantly higher positive charge than those of slow-replicating, non-syncytium-inducing monocytotropic isolates. It appeared that these differences in charge could be attributed to highly variable amino acid residues located on either side of the V3 loop, midway between the cysteine residues and the central GPG motif. In non-syncytium-inducing monocytotropic isolates, these residues were negatively charged or uncharged, whereas in syncytium-inducing nonmonocytotropic isolates, either one or both were positively charged. The substitutions at these positions result in changes in the predicted secondary structure of the V3 loop. Our data suggest that two amino acid residues in the highly variable V3 domain are responsible for phenotype differences and point to conformational differences in V3 loops from phenotypically distinct HIV-1 isolates.

Previously, a correlation between the biological phenotype of a human immunodeficiency virus type 1 (HIV-1) isolate and the clinical course of infection was demonstrated (33). Fast-replicating, syncytium-inducing (SI) variants emerge in 50% of HIV-1-seropositive individuals preceding progression to AIDS. Slow-replicating, non-syncytium-inducing (NSI) variants are predominant in the asymptomatic stage and persist throughout all stages of HIV-1 infection (33). The NSI isolates in general are much more monocytotropic and probably important for persistence, possibly by forming a major viral reservoir in HIV-1 infection (11, 25, 26). By using a limited set of viral isolates, regions in the envelope gene have been delineated that are involved in determining host range, and in all cases, involvement of the V3 domain has been suggested (4, 14, 20, 28, 36).

The V3 domain of HIV-1 gp120 has been found to elicit neutralizing antibodies as well as a cytotoxic and helper T-cell response in both humans and animals (7, 12, 15, 16, 21, 23, 30). As a consequence, this immunodominant principal neutralizing determinant has become an important target for vaccine development. Type specificity has been observed in both antibody and T-cell responses as a result of variation in V3 sequences (1, 21, 31), which has hampered development of an effective vaccine for HIV-1.

The aim of our study was to investigate whether the primary or predicted secondary structure of the V3 loop correlates with differences in biological phenotype of HIV-1 variants. For this purpose, a large panel of primary virus isolates and biological clones were used that were well defined for their capacity to induce syncytia and to replicate in primary monocytes (26, 27, 33).

DNA was isolated from infected cells as described before (2). V3 sequences were amplified by the polymerase chain reaction with primers A and H in the first and B and C in the second reaction, as described elsewhere (29). Products were purified with a Geneclean kit (Bio 101), and both strands were sequenced directly with primers B and C by the dideoxy chain termination method with Sequenase (USB), both according to instructions from the manufacturers.

In Fig. 1 and 2, the derived amino acid sequences of the V3 loops of the virus isolates are shown, aligned with the consensus sequence of the isolates. The most extensive variation is observed in the regions flanking the GPG sequence at amino acids 10 to 14 and 25 to 29. In addition, sequence variation is higher in the group of SI nonmonocytotropic isolates than in the NSI monocytotropic isolates.

It has been suggested that the relative charge of the V3 domain might influence its function or the function of the entire gp120 molecule, for example, in binding negatively charged counterpart structures (3). Using the CHARGEPRO program from PCGENE (IntelliGenetics), we calculated the charge of the V3 domains at physiological pH. NSI isolates had less positively charged V3 amino acid sequences than the SI isolates (3.6 ± 0.6 [standard deviation] versus 5.5 ± 1.3, $P < 0.0001$) (Fig. 3A). Similar results were obtained when comparing monocytotropic and non-monocytotropic isolates (3.6 ± 0.7 versus 4.9 ± 1.2, $P = 0.007$) (Fig. 3B).

To investigate whether substitution of neutral or acidic residues for positively charged residues required fixed positions, statistical discriminant analysis was performed after assigning numbers to amino acid residues based on charge (acid, neutral, and basic residues were numbered 1, 2, and 3, respectively). By use of this analysis, NSI and SI HIV-1 variants as well as monocytotropic and nonmonocytotropic HIV-1 variants were compared. The serine and glutamic acid residues at positions 11 and 28 of the consensus sequence discriminated between different phenotypes of virus isolates on the basis of their primary V3 structure. In NSI variants, the amino acid residue at position 11 was uncharged (in

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HIV ISOLATE	SI	Mono-cyto-trop.	1 10 20 30 38 CTRPNNNTRKSI.HI..GPGRAFYTTGEIIGDIRQAHC
Ams-55	+	-	--------K-G-.AV..----DI--ADK----LK----
Ams-32	+	-	-----S------.R-HR----------M----------
Ams-42	+	-	-------IKRR-I---..------HA--G-.--------
ACH-320.2A.5	+	-	----------G-.--..-------AARK----------
ACH-320.2A.7	+	-	----------G-.--..-------AARK----------
ACH-320.2A.1.1	+	-	----------G-.--..-------AARK----------
Ams-16.1	+	-	----------G-.--..-----V----R----------
Ams-16.2	+	-	----------G-.--..-----V----R----------
ACH-168.7	+	-	----------R-.--..----------Q---N------
ACH-168.10	+	-	----------R-.--..----------Q---N------
ACH-479.5	+	-	---------QG-.--..---------RR----------
ACH-479.6	+	-	---------QG-.--..---------RR----------
ACH-373.38.5	+	-	-------I-RRMI---..----------------N------
ACH-704.1	+	-	----------RV.TM..-----L----------------
Ams-127.4.1	+	-	----------RV.TM..----VL----------K----
Ams-169.1	+	-	----------G-RIGHI----V-----K----------
Ams-175	+	-	---------RG-.Y-..-----V--K-R----------
Ams-127.4.2	+	+/-	----------RV.TM..----VL----------K----
Ams-169.2	+	+/-	----------G-HIGHI----V-----K----------
Ams-37	+	+/-	----------RV.TL..----VW----------K-Y-
HTLVIIIB	+	+/-	----------R-.R-QR------V-I-K-.-NM-----
ACH-182.69	+	+/-	---LSA-KIRRM.--..-------A--K------K---
ACH-479.7	+	+/-	----------QG-.--..---------RR----------
ACH-479.8	+	+/-	----------QG-.Y-..---------RR----------
Ams-165.1	+	NT	---LS----RGVHIKHI----V-----AV-----R---
Ams-164.1	+	NT	-----R-KIRR-.--..----P--G-.D-E-P----Y-
Ams-119.1	-	-	------------.--..A--S---A--A------K---
Ams-96.1	-	-	---------R--.--..------FG-.D---N------
Ams-168.1	-	-	------------.N-..-------A--D----------
ACH-168.1	-	+/-	----------G-.-M..-------A--D----------
ACH-168.2	-	+/-	------------.--..-------A--D----------
ACH-373.38.3	-	+/-	------------.--..----------N------
ACH-373.38.4	-	+/-	------------.--..----------N------
Ams-169.3	-	+/-	----------G-.--..-------A----------
ACH-15.9	-	+/-	------------.NM..-----I----Q-V--------
Ams-176.BM	-	+/-	------------.N-..-------A----------
ACH-239.11	-	+/-	----S-------.--..-------A--D----------
Ams-119.2	-	+/-	------------.--..A--S---A--A------K---
Ams-96.2	-	+/-	---------RG-.--..-------G-.D---N------
ACH-182.6	-	+/-	------------.N-..-------A--A------R---
Ams-168.2	-	+/-	------------.N-..-------A--D----------
ACH-320.2A.2.1	-	+	----------G-.--..---K---A--Q----------
ACH-320.2A.3	-	+	----------G-.-M..---K---A--Q----------
Ams-24	-	+	------------.PM..----------D----------
ACH-172.Ba-L	-	+	-I----------.-M..-------A----------
HTLVIIIB.Ba-L	-	+	------------.--..-----L----------
ACH-63.11	-	+	------------.N-..-------A--A----------
Ams-44	-	+	------------.-L..---K-----D----------
Ams-101	-	+	------------.--..-----V----------
Ams-119.MDM	-	+	---------R--.--..A------A--A------K---
Ams-96.MDM	-	+	----H----RG-.--..----------S-T--------
Ams-165.2	-	NT	---LS----RGV.--..----------AV---------
Ams-164.2	-	NT	-------K-Q--.--..------FG-.D---N----Y-
Ams-16.3	-	NT	----------G-.--..----------

FIG. 1. Alignment of V3 sequences of different isolates with the consensus sequence. Gaps are indicated as points; similarity to the consensus sequence is shown as dashes. The syncytium-inducing capacity (SI) and monocytotropism of the viral isolates are indicated (isolates were tested on monocyte-derived macrophages from eight seronegative donors). For monocytotropism, − represents replication in 0, + represents replication in ≥4, and +/− represents replication in the monocytes of 1 to 3 of the donors tested; N.T., not tested. Amino acid residues in boldface were used as classification variables for statistical analysis (Table 1).

PATIENT	SI	Mono-cyto-trop.	1 10 20 30 38 CTRPNNNTRKSI.HI..GPGRAFYTTGEIIGDIRQAHC
ACH-182	-	+/-	------------.N-..-------A--A------R---
	+	+/-	---LSA-KIRRM.--..-------A--K------K---
ACH-320	-	+	----------G-.-M..---K---A--Q----------
	+	-	----------G-.--..-------AARK----------
Ams-16	-	NT	----------G-.--..----------
	+	-	----------G-.--..-----V----R----------
ACH-168	-	+/-	------------.--..-------A--D----------
	+	-	----------R-.--..----------Q---N------
ACH-479	-	-	----------G-.N-..-------A--Q---N------
	+	+/-	---------QG-.--..---------RR----------
ACH-373	-	+/-	------------.--..----------N------
	+	-	-------I-RRMI---..----------N------
Ams-169	-	+/-	----------G-.--..-------A----------
	+	-	----------G-HIGHI----V-----K----------
Ams-119	-	+	---------R--.--..A------A--A------K---
	-	-	------------.--..A--S---A--A------K---
Ams-96	-	+	----H----RG-.--..----------S-T--------
	-	-	---------R--.--..------FG-.D---N------
Ams-168	-	+/-	------------.N-..-------A--D----------
	-	-	------------.N-..-------A--D----------
Ams-165	-	NT	---LS----RGV.--..----------AV---------
	+	NT	---LS----RGVHIKHI----V-----AV-----R---
Ams-164	-	NT	-------K-Q--.--..------FG-.D---N----Y-
	+	NT	-----R-KIRR-.--..----P--G-.D-E-P----Y-

FIG. 2. Sequence alignment of biological clones with distinct phenotypes obtained from the same individual. See legend to Fig. 1 for details.

general, S or G) and the residue at position 28 was either negatively charged (E or D) or uncharged (A or Q). In SI isolates, either one or both amino acid residues at positions 11 and 28 were replaced by basic residues ($P < 0.00001$; Table 1). Comparison of monocytotropic and nonmonocytotropic isolates revealed identical variation, albeit less pronounced ($P = 0.0006$; Table 1). It should be noted that in this set of isolates, the occurrence of a basic residue at position 28 coincides with a glycine residue at position 11 in all isolates. NSI monocytotropic isolates with a glycine residue at position 11 could therefore be an intermediate genotype in populations of viruses during phenotype conversions. Other changes in the V3 sequences occurred in both groups of virus isolates or occurred infrequently and were therefore neither predictive nor discriminative. In contrast to the findings by Westervelt et al. (34), no discriminatory value for the amino acid residues at positions 14 and 24 was observed.

If amino acid residues 11 and 28 are the major determinants of the viral phenotypes, comparable changes should be present in highly related yet phenotypically distinct variants, such as those present in sequential isolates from seropositive individuals (33). In Fig. 2, the V3 sequences of sets of biological clones obtained from 12 seropositive individuals are shown. In these highly related viruses, similar changes of amino acid residues at position 11 and/or 28 to positive residues upon transition from NSI to SI can be demonstrated. The specific variation at positions 11 and 28 upon transition suggests an important contribution of these amino acid residues to determining the biological phenotype of the isolates.

Whether these amino acid substitutions could cause changes in the V3 secondary structure was investigated by use of the method of Garnier (10a) (PCGENE; IntelliGenetics). Substitution of the residues at positions 11 and 28 with basic residues appears to induce changes in secondary structure at the tip of the loop (Fig. 4), indicating that NSI monocytotropic and SI nonmonocytotropic isolates might have distinct V3 structures.

The fusion capacity-associated sequence variation in these viral isolates points to a prominent role for the V3 domain in determining this biological property. However, analysis of chimeric proviruses has revealed that V3 is involved in, but not sufficient for, determining monocytotropism (5, 14, 28, 34). The fact that sequence variation related to the monocytotropic and NSI phenotypes is comparable could be due to an 80% overlap of these virus populations (26). A proper combination of V3 and other functional domains involved in determining monocytotropism appears to lead to the monocytotropic phenotype (14). V3 sequences from the highly related viral isolates presented in Fig. 2 point to a minor role for amino acid variation at positions 11 and 28 in determining monocytotropism (compare Ams-96, Ams-119, and Ams-168).

If the correlation between V3 sequence and viral phenotype is based on a structure-function relation, several mechanisms can be suggested. For instance, the charge and secondary structure of the V3 loop might influence binding of the virion, resulting in altered syncytium induction and infectivity. In addition, processes subsequent to exposure of

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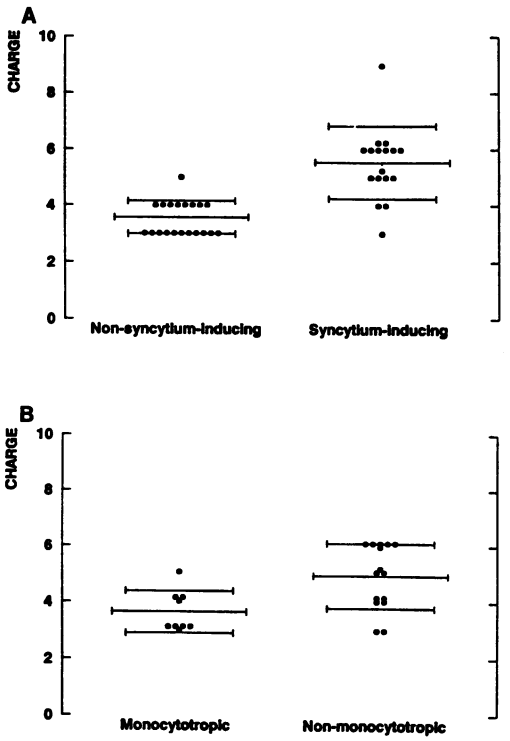

FIG. 3. Comparison of the peptide charges at pH 7.0 as calculated by use of the CHARGEPRO program from PCGENE (IntelliGenetics). (A) Comparison on the basis of syncytium-inducing capacity; (B) comparison on the basis of monocytotropism. Note: multiple biological HIV-1 clones obtained from one individual with identical cytotropism or syncytium-inducing capacity were excluded from this analysis to prevent a bias in the results.

the V3 domain upon binding of gp120 to CD4 might be influenced (24). Site-directed mutagenesis has indicated that the V3 domain plays a role in the syncytium-inducing capacity and infectivity of virus isolates (10, 32) at the level of viral fusogenic capacity. In a recent study, a limited number of chimeric proviruses were constructed that differed only in the regions that contained the critical amino acid residues at positions 11 and 28. These chimeras demonstrated correlated differences in syncytium-inducing capacity, pointing to the functional importance of these residues (8). Furthermore, syncytium-inducing capacity has been demonstrated to be dependent on V3 conformation (17). In addition, it has been suggested that V3 is cleaved by proteases, which is a conformation-dependent process (6,

PRIMARY PND STRUCTURE	PREDICTED SECONDARY PND STRUCTURE
CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
----------	EECTTTTTEEEEEEECTCEEEEEEEEEEHHHHHEE
----------R----------	----------C----------
----------R----------	----------E-----T----EEE-----
----------K----------	----------E-----T----EEEEE--
----------R----------K----------	----------C--------T----EEE-----
----------H----------K----------	----------C--------T----EEE-----

FIG. 4. Predicted secondary structures of V3 sequences after substituting basic residues at position 11 and/or 28, as occurring in the primary virus isolates, by the method of Garnier (10a). H, helix; C, coil; T, turn; E, extended configuration. PND, principal neutralizing determinant.

TABLE 1. Comparison of the charge of amino acid residues 11 and 28

Isolates	Charge[a] (no. of isolates)	
	Positive	Negative or none
Syncytium induction		
SI	16	1
NSI	0	20
Tropism		
Nonmonocytotropic	11	4
Monocytotropic	0	9

[a] Syncytium induction and monocytotropism were both significantly correlated with charge ($P < 0.00001$ and $P = 0.0006$, respectively).

13). Therefore, V3 secondary structure might be involved in determining the biological properties of the gp120 molecule.

The Los Alamos V3 consensus sequence (19) is derived mainly from direct or singly passaged material. Since the predominant phenotype of the viral isolates involved in persistent infection is NSI monocytotropic (25), one may expect the consensus sequence to resemble the V3 sequence of this virus phenotype. Indeed, V3 sequences obtained from the brain and spleen of HIV-1-infected individuals as well as NSI monocytotropic isolates show high homology to the consensus sequence (9). The high homology to the consensus V3 in the NSI monocytotropic viral isolates supports the suggestion that these variants constitute the virus tissue reservoir.

Since both SI and NSI variants can be transmitted (22), it appears that the uncompromised immune system is well able to eradicate or suppress fast-replicating SI variants upon primary infection, in contrast to slow-replicating NSI variants, which may not be as immunogenic, possibly by virtue of their monocytotropism. Because of the persistence of the NSI monocytotropic variants (26), these variants should be an important target for vaccine development.

In conclusion, we have demonstrated that sequence variation at positions 11 and 28 in V3 correlates with biological variation in HIV-1. The functional involvement of envelope proteins in processes such as fusion is thought to be highly dependent on protein conformation, directly or indirectly determined by V3 sequences (17). The conformational differences between HIV-1 variants may result in differential antibody recognition, relevant for induction of neutralizing antibody responses. Indeed, differential susceptibility of sequential HIV-1 isolates for neutralization with autologous sera has been demonstrated, pointing to different antigenicities of V3 (1). Moreover, it was shown that naturally occurring, single-amino-acid-residue substitutions affect the antigenicity of V3 (18, 35). Therefore, it may be desirable for efficacious AIDS vaccines to include V3 sequences from both the persistent NSI monocytotropic variants and the pathogenic SI nonmonocytotropic variants to induce neutralizing antibodies and T-cell responses to both biological variants.

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