Molecular determinants of the V3 loop of human immunodeficiency virus type 1 glycoprotein gp120 responsible for controlling cell tropism

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We and others have identified the major determinant of cell tropism in human immunodeficiency virus type 1 (HIV-1) as the V3 loop of glycoprotein gp120. We have conducted a detailed study of two molecularly cloned isolates of HIV-1, HIV Jr-csf and HIV Nl4-3, that differ in their tropism for immortalized CD4+ cell lines, by constructing a series of site-directed mutations within the V3 loop of HIV Jr-csf based on the sequence of HIV Nl4-3. The phenotypes of these mutants fall into two classes, those which are viable and those which are not. A spontaneous mutant with significantly altered growth properties was also recovered and found to have an additional single amino acid change in the V3 loop sequence. The carboxy-terminal β-strand part of the V3 loop is the major determinant of cell tropism. However, the results presented here indicate that the functional role of the V3 loop sequences can only be interpreted properly in the context of the original gp120 backbone from which they were derived. These findings show that over-simplistic interpretation of sequence data derived from unknown mixtures of HIV variants in infected persons may be highly misleading.

Human immunodeficiency virus (HIV) isolates HIV Jr-csf (Koyanagi et al., 1987) and HIV Nl4-3 (Adachi et al., 1986) differ in their tropism for immortalized CD4+ cell lines. HIV Nl4-3 replicates in both primary human peripheral blood lymphocytes (PBL) and some immortalized CD4+ cell lines whereas replication of HIV Jr-csf is restricted to PBL (Cann et al., 1990, 1992; Chesebro et al., 1991). We have previously identified the major determinant of cell tropism in HIV Jr-csf as the V3 loop of glycoprotein gp120 (Cann et al., 1992; Chesebro et al., 1991). Similar observations on the importance of V3 have also been made for other isolates of HIV-1 (Cheng-Mayer et al., 1990; Hwang et al., 1991; Liu et al., 1990; O'Brien et al., 1990; Shioda et al., 1991; York-Higgins et al., 1990).

Our earlier experiments were based on the construction of recombinant provirus genomes by means of substituting restriction endonuclease fragments between infectious molecular clones of HIV Jr-csf and HIV Nl4-3. The conclusion of these experiments was that the V3 loop of gp120 determines the cell tropism of these isolates (Cann et al., 1992; Chesebro et al., 1991). To refine these studies we have constructed a series of site-directed mutations within the V3 loop of HIV Jr-csf. This series of mutations defines which residues in the V3 loop are important in controlling the tropism of HIV-1 and which alterations can be made without affecting the growth of the virus.

All virus infections were performed with virus stocks normalized for p24 antigen concentration as described previously (Cann et al., 1990). Primary human PBL were collected, stimulated for 48 to 72 h with phytohaemagglutinin and then cultured in RPMI 1640 medium containing 20% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), 2 mM-glutamine and recombinant interleukin-2 (5 U/ml) as described previously (Cann et al., 1990). The immortalised T cell lines HUT78, Molt 4/8 and SUPT1 were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and 2 mM-glutamine. The p24 (gag) antigen titres in the supernatant of HIV infected cultures were measured by quantitative p24 capture ELISA.

All of the alterations in the V3 loop described in this paper were created in a circularly permuted clone of HIV Jr-csf (Cann, 1990). The HindIII(6574)–BamHI(7324) fragment of this clone containing the V1, V2 and V3 regions was subcloned into M13mp18. An efficient oligonucleotide-directed mutagenesis method was used to change selected amino acid residues in the HIV Jr-csf V3 loop to resemble those in the equivalent
Table 1. Amino acid sequences of V3 loop mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Residues</th>
<th>M_r</th>
<th>Charge</th>
<th>Tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable V3 mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JR-CSF</td>
<td>CTRPSNNTRKS1HI...GPGRAFYTTGEIGDIRQAHC</td>
<td>35</td>
<td>3868</td>
<td>+5</td>
<td>JR-CSF</td>
</tr>
<tr>
<td>M1/M2 (= NL4-3)</td>
<td>.......N........R.QR........V.I.AK.GNM......</td>
<td>36</td>
<td>4050</td>
<td>+9</td>
<td>NL4-3</td>
</tr>
<tr>
<td>M1</td>
<td>.......N........R.QR........</td>
<td>37</td>
<td>4199</td>
<td>+6</td>
<td>JR-CSF</td>
</tr>
<tr>
<td>M1*</td>
<td></td>
<td>37</td>
<td>4127</td>
<td>+7</td>
<td>NL4-3</td>
</tr>
<tr>
<td>M12</td>
<td></td>
<td>35</td>
<td>3834</td>
<td>+5</td>
<td>JR-CSF</td>
</tr>
<tr>
<td>Non-viable V3 mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(JR-CSF)</td>
<td>CTRPSNNTRKS1HI...GPGRAFYTTGEIGDIRQAHC</td>
<td>34</td>
<td>3719</td>
<td>+8</td>
<td>?</td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td>34</td>
<td>3719</td>
<td>+8</td>
<td>?</td>
</tr>
<tr>
<td>M3</td>
<td></td>
<td>35</td>
<td>3858</td>
<td>+5</td>
<td>?</td>
</tr>
<tr>
<td>M10</td>
<td></td>
<td>37</td>
<td>4153</td>
<td>+6</td>
<td>?</td>
</tr>
<tr>
<td>M11</td>
<td></td>
<td>34</td>
<td>3754</td>
<td>+7</td>
<td>?</td>
</tr>
</tbody>
</table>

position in the HIV_{NL4-3} V3 loop (Venkitaraman, 1989) (Fig. 1, Table 1). In addition, the sequence alteration in mutant M12 has been reported to increase the affinity of gp120 for CD4 (Hwang et al., 1992). Mutant M13 plagues were selected by single lane tracking and the entire HindIII-BamHI region was sequenced. Replicative form DNA of the mutated M13 clones was prepared and the 722 bp DraIII-BamHI fragments were inserted back into the circularly permuted HIV_{JR-CSF} provirus clone. All recombinant permuted clones were rechecked by direct sequencing of the plasmid dsDNA before electroporation. Samples (25 μg) of each caesium chloride gradient-purified plasmid DNA were digested and religated at high concentration to form concatemers and these were used for electroporation of PBL as previously described (Cann, 1990). Virus production was detected by assays of 0.45 μm-filtered culture supernatants conducted at 3 to 4 day intervals by p24 ELISA. The identity of all rescued viruses was confirmed by PCR amplification and sequencing of extracted cellular DNA. Each experiment was repeated several (2 to 4) times. Results shown are from a single representative experiment.

All experiments were performed on virus stocks rescued from molecularly cloned DNA. The phenotypes of the mutants fall into two classes, those which are viable (M1, M1/M2 and M12) and those which are not (M2, M3, M10, M11) (Table 1). All of the viable mutants were rescued at the first attempt. In the case of the non-viable mutants, the entire 722 bp DraIII-BamHI region from the mutated M13 clones was sequenced before being inserted into a batch of HIV_{JR-CSF} DNA known to give rise to infectious virus. For each of these mutants, three independent attempts were made to rescue virus derived from between three and six different M13 clones (with appropriate positive and negative controls in each of these experiments). Thus we are entirely confident that this second group of mutations (M2, M3, M10, M11) cannot be tolerated within the gp120 backbone of HIV_{JR-CSF}.
Fig. 2. Phenotypes (cell tropism) of rescued viruses compared with those of the HIVJR-CSF and HIVNL4-3 parental isolates. ○, PBL: JR-CSF; □, PBL: NL4-3, ●, HUT78: JR-CSF; ▼, HUT78: NL4-3. See Methods for details of infections and assay methods. Results shown are from a single representative experiment: (a) mutant M1 (△, PBL: M1; ▲, HUT78: M1); (b) mutant M1/M2 (△, PBL: M1/M2; ▲, HUT78: M1/M2); (c) mutant M12 (△, PBL: M12; ▲, HUT78: M12); and (d) mutant M1* (△, PBL: M1*; ▲, HUT78: M1*).

Table 2. p24 titre (ng/ml) on day 15 after infection

<table>
<thead>
<tr>
<th></th>
<th>PBL</th>
<th>HUT78</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR-CSF</td>
<td>1·9</td>
<td>0</td>
</tr>
<tr>
<td>NL4-3</td>
<td>2·53</td>
<td>2·98</td>
</tr>
<tr>
<td>Mutant M1</td>
<td>0·55</td>
<td>0</td>
</tr>
<tr>
<td>Mutant M1/M2</td>
<td>2·25</td>
<td>1·6</td>
</tr>
<tr>
<td>Mutant M12</td>
<td>2·02</td>
<td>0</td>
</tr>
<tr>
<td>Mutant M1*</td>
<td>3·0</td>
<td>2·6</td>
</tr>
</tbody>
</table>

The phenotypes of the viable mutants as compared with the HIVJR-CSF and HIVNL4-3 parental isolates are shown in Fig. 2. These data are also summarized in Table 2. The phenotype of mutant M1 is similar to that of HIVJR-CSF (Fig. 2a). Thus, substitution of sequences in the NH₂-terminal part of the V3 loop do not alter the cell tropism of this virus. The phenotype of mutant M12 is also similar to that of HIVJR-CSF (Fig. 2c). Mutant M1/M2 contains the entire V3 loop of HIVNL4-3 gp120 and the phenotype of this virus is similar to that of HIVNL4-3 (Fig. 2b), in agreement with our previously published results (Cann et al., 1992; Chesebro et al., 1991). In addition to HUT78 cells, the M1/M2 mutant is also capable of growing in certain other immortalized CD4⁺ cell lines, such as Molt 4 and SUPT1 (data not shown), as is HIVNL4-3. This finding is consistent with our earlier published results (Cann et al., 1992; Chesebro et al., 1991). Clearly, it is not possible to comment on the phenotype of non-viable mutants (see below).

Mutant M1* was rescued from an attempt to infect HUT78 cells with mutant M1. M1* contains a single nucleotide change encoding an amino acid substitution (E → G₃₁₈) in addition to the V3 sequences present in mutant M1. This spontaneous mutant has altered cell tropism from that of either HIVJR-CSF or mutant M1 and resembles HIVNL4-3 (Fig. 2d).

Comparison of the amino acid sequences of the V3 loop of HIVJR-CSF and HIVNL4-3 shows nine coding
differences, as shown in Fig. 1. We have systematically changed these residues alone and in various combinations by site-directed mutagenesis, as described above. Analysis of the viable and non-viable groups of mutants fails to reveal any consistent differences between them that would explain why some give rise to infectious viruses whereas the others do not. Recent structural studies indicate that the GPGR sequence at the tip of the V3 loop forms a type II \( \beta \)-turn and is flanked by hypervariable \( \beta \)-strand regions (Ghiara et al., 1994). The phenotype of all the viable mutants resembles the HIV\( \text{JR-CSF} \) parent virus, except that of M1/M2 which contains the entire V3 loop of HIV\( \text{NL4-3} \). Thus we have defined certain regions of the V3 loop which do not appear to affect the cell tropism of this isolate i.e. the amino-terminal \( \beta \)-strand part of the loop. The carboxy-terminal \( \beta \)-strand part of the V3 loop thus appears to be the major determinant of cell tropism. This is in agreement with results published by other groups (see below).

Our attempts to analyse the phenotype of the other mutations in the carboxy-terminal region have been frustrated by the lack of infectivity of the resulting viruses. We are thus forced to conclude that the precise combinations of amino acids in the V3 loops of these non-viable mutants are not tolerated by this virus (HIV\( \text{JR-CSF} \)). These data clearly show that there are limits on the sequence variation which is allowable in V3 without corresponding and compensating variation in the other variable domains of gp120. We have analysed common features of the V3 sequence of HIV\( \text{JR-CSF} \) gp120 and the mutants that might determine what features of the sequence are 'allowable', including the number of amino acid residues, overall size (\( M_r \)), charge and hydropathy of the loop sequence. We can identify no obvious single characteristic that appears to distinguish between the viable (i.e. replication competent) and non-viable proteins analysed. On the one hand, this is a somewhat surprising finding, since similar or identical V3 sequences to some of these mutants have been published by other groups (De Jong et al., 1992; Hwang et al., 1991, 1992; Shioda et al., 1991; Takeuchi et al., 1991). However, these published V3 sequences are all expressed in the context of different gp120 backbones. It has been shown that identical amino acid changes in the V3 region of different HIV-1 isolates do not necessarily have the same effect on the phenotype of all isolates (Page et al., 1992). Moreover, results that we have obtained previously clearly indicate that long-range structural interactions between widely separated regions of gp120 affect the functionality of the protein (Boyd et al., 1993) (see below).

Based on these results and on the published results of others (e.g. Milich et al., 1993), we predict that the carboxyl \( \beta \)-strand of the V3 loop containing the acidic sequence ‘GEII’ is vital in determining the cell tropism of HIV-1. This is confirmed by the sequence of a spontaneous mutant of M1 (M1\( \ast \)) (Table 1, Figure 2d). This virus was recovered from HUT78 cells infected with mutant M1. When the V3 loop region was sequenced, a single nucleotide change encoding an amino acid substitution (E \( \rightarrow \) G) was discovered in addition to the alteration of the NH\(_2\)-terminal \( \beta \)-strand in M1, which does not in itself change the cell tropism of the M1 mutant. This observation also points to the significance of the carboxy-terminal \( \beta \)-strand region of the V3 loop.

We attempted to measure the affinity of gp120 from the parental and mutant viruses for CD4 by various assays. Attempts to utilize CD4-binding ELISA and gel filtration methods failed because the HIV\( \text{JR-CSF} \) isolate and mutants based on this virus do not grow to sufficiently high titres to permit these tests to be used successfully (M. Boyd, personal communication). Investigating neutralization of virus infectivity by sCD4, we have not been able to demonstrate any conclusive differences between any of the viruses described above. We have also examined the phenotype of two mutations reported to alter the affinity of gp120 for CD4, I \( \rightarrow \) F\( _{115} \) (Cordonnier et al., 1989) (data not shown) and A \( \rightarrow \) L\( _{233} \) in mutant M12 (Hwang et al., 1992). Neither of these mutations altered the phenotype of HIV\( \text{JR-CSF} \). It has been established that mutations in V3 can give rise to non-viable viruses that can still bind CD4 (Freed et al., 1991; Grimalia et al., 1992; Helseth et al., 1990; Page et al., 1992). Thus, we conclude that the restricted cell tropism of HIV\( \text{JR-CSF} \) is not determined by the relative affinity of gp120 for CD4.

In addition to V3, we have previously shown that alteration of the V1 loop of gp120 is also capable of changing the cell tropism of HIV-1 (Boyd et al., 1993). This observation is in accord with other studies that indicate that although V3 is the major determinant of cell tropism in HIV-1, other regions of the protein such as V1 and C2 (Stamatatos & Cheng-Meyer, 1993) also have a role to play. Therefore, the phenotypic effect of individual mutations in variable regions of HIV-1 gp120 can only be interpreted in the context of the original, entire gp120 sequence, due to long-range structural interactions between widely separated regions of gp120 that affect the functionality of the protein.

Recently, a number of studies have attempted to interpret the function of V3 loop sequences derived by PCR from complex mixtures of strains in HIV-infected patients, without any reference to the sequence of the rest of the gp120 backbone from which these loop sequences were derived (e.g. Foucher et al., 1992; Milich et al., 1993; Ball et al., 1994). Whereas the data presented here agree with these reports on the significance of the
carboxyl-terminal $\beta$-strand of the V3 loop in determining the tropism of HIV-1, we conclude that over-interpretation of limited PCR sequence data in the absence of any information concerning the rest of gp120 may be highly misleading.

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References


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