Is the V3 Loop Involved in HIV Binding to CD4?†

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ABSTRACT: The entry of the human immunodeficiency virus into cells requires the interaction of the viral envelope glycoprotein gp120 with CD4 and a chemokine receptor. The gp120 binding site has been previously mapped to the Ig-CDR2-like region of CD4 first domain. A second area of this domain (Ig-CDR3-like region) is involved in gp120−CD4 interactions, but its gp120 counterpart remained so far unknown. Using a photoaffinity labeling experiment, we demonstrate that a peptide, mapping the (307−330)m region of HIV-MN-gp120 V3 loop, binds a sequence including a part of the Ig-CDR3-like region. These results may contribute to explain the complex mechanism of human immunodeficiency virus penetration, helping the development of new therapeutic agents.

The HIV-1[1] and HIV-2 cause the destruction of $CD4^+$ cells in their hosts, resulting in the development of AIDS (*1*). Entry of HIV into target cells generally requires the interaction of the envelope glycoprotein, gp120, with the amino-terminal region of the four immunoglobulin-like domains of CD4, and a chemokine receptor (*2*−*4*). The structure of the N-terminal domain (*5*) and of the entire extracellular portion of CD4 (*6*) have been determined. Mutagenesis investigations (*7*, *8*) and the X-ray crystal structure of a HIV-1 gp120 core complexed with a two domains fragment of human CD4 and a fragment of a neutralizing antibody (*9*) indicate that the second immunoglobulin-like region (Ig-CDR2-like region) of CD4 is critical for gp120 binding (*7*, *8*). The CD4 binding site on gp120 is represented by a depression formed at the interface of the three principal parts of the protein, named inner domain, outer domain, and bridging sheet (*9*). Contact residues are concentrated from 25 to 64 in CD4, but they are distributed over six segments in gp120 (*9*). All naturally occurring isolates of HIV-1 require, in addition to the CD4 molecule, a chemokine receptor, usually CXCR4 or CCR5, for viral entry (*10*, *11*). Multiple extracellular regions of the coreceptors are involved in the interaction with gp120, and depending upon the envelope protein, the critical extracellular domains vary (*12*−*15*). Several studies of envelope determinants of coreceptor utilization have shown that mutations in gp120 alter tropism. The V3 loop has long been recognized as the principal determinant of viral tropism, and it has now been demonstrated that substitution of the entire V3 loop, or just single residues within it, can result in a switch in coreceptor utilization (*16*−*19*). Specific amino acid substitutions within the V3 loop resulted in loss of infectivity, host range and syncytium forming potential of HIV (*20*). The current interpretation of these data is that a functional interaction must occur between the V3 region and the different coreceptors. However, to date, no attempt (*21*, *22*) has allowed a formal demonstration of a physical interaction between the V3 peptides and the cognate coreceptor. We have previously demonstrated that a synthetic peptide reproducing the PND of the HIV-1-MN strain (DB3) is able to bind CD4 molecule at the V1/V2 domain site *(23)*. In fact, the interaction between DB3 and sCD4-matrix has been drastically inhibited by addition of MT151 or B66.6.1 anti-CD4 mAbs, while competition with anti-Leu3a mAb has not been observed. In addition, we have demonstrated that DB3 enhances HIV-1 induced syncytium formation and infection in $CD4^+$ target cells (*24*) through a dose-dependent and not strain-restricted mechanism. Cultures treated with DB3 exhibit large syncytia within 48 h post infection, whereas untreated cells show only small syncytia up to 4 days (*24*): this effect has been utilized as the basis of a method for the direct in vitro diagnosis of HIV-1 infections; the method is particularly useful to detect the presence of HIV-1 in the early stages of the infection, i.e., when the patients have not been infected long enough to produce anti-HIV antibodies (*25*−*27*). Point-mutation investigations revealed that Phe^{15} → Ile, Tyr^{16} → Ile, and His^8 → D-His substitutions completely abrogate the activity of the peptide; Lys^3 → Asn and Arg^6 → Asn analogues had a lower enhancing effect, whereas Lys^{19} → Asn analogue showed the strongest enhancement of HIV-1 infection (*28*). Additional investigations strongly suggested that DB3/CD4 interaction induces an increase in both CD4 expression and CD4/gp120 binding affinity, which, in turn, mediates the enhancement of viral infection (*28*).

To add confirmatory evidence to the hypothesis of the existence of a specific interaction between the V3 loop of gp120 and CD4 and to identify the exact binding site of DB3 on CD4, we have now designed a photoaffinity labeling experiment.

Photoaffinity labeling is a very potent method for identification and isolation of bioactive peptide receptors. In this

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[1] Abbreviations: AIDS, acquired immuno deficiency sindrome; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PND, principal neutralizing domain; PSD, post-source decay; TFA, trifluoroacetic acid.

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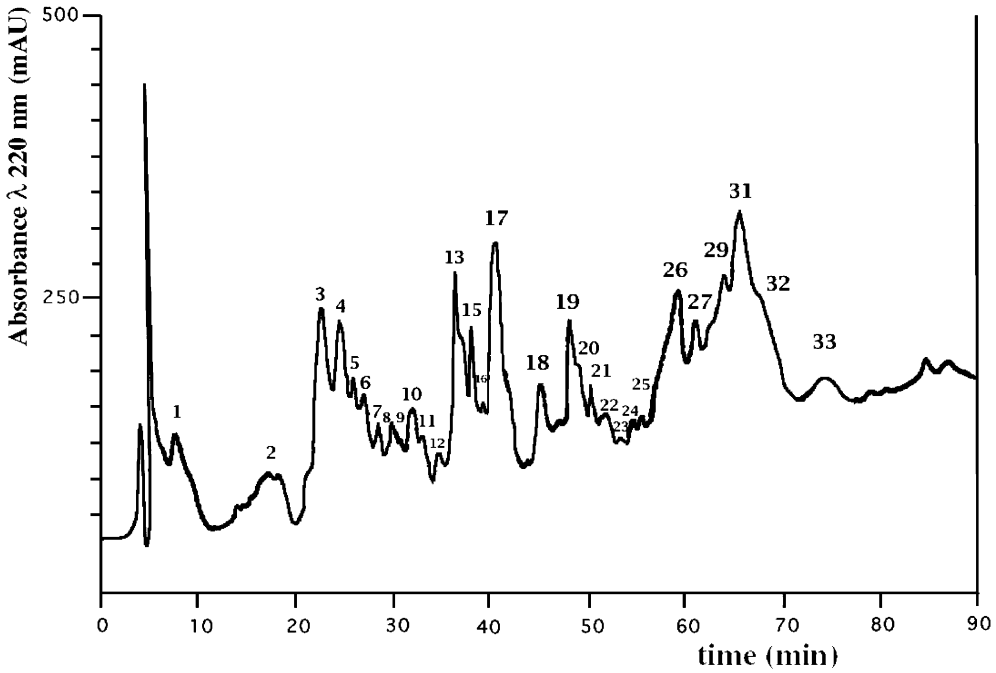

FIGURE 1: HPLC analysis of the endoproteinase Asp-N digest of the covalent complex CD4−DB3. The peptide mixture has been analyzed using a Vydac C_{18} column (250 × 2.1 mm, 5 μm), as described in the Methods section, and the peptides have been collected for mass analysis. Peak identification is reported in Table 1.

approach, a chemically stable but photolabile group is conjugated to a potent ligand. After binding of this ligand to its receptor site, the receptor−ligand complex is subjected to photolysis to generate highly reactive carbenes or nitrenes that may undergo hydrogen abstraction, insertion, or cycloaddition reactions with the adjacent chemical functionalities on the receptor molecule, thereby establishing a covalent bond between the receptor and the ligand (*29*). Accordingly, two DB3 analogues, carrying different photoaffinity markers in the side-chain of the first residue, have been incubated with a soluble form of CD4 (sCD4) and irradiated to produce the formation of a DB3−CD4 covalent complex (*30*). The characterization of the covalent complex by enzymatic digestion fragments analysis and the consequent identification of HIV-1-MN gp120 PND binding site on sCD4 reported here add experimental evidence to the hypothesis of V3 loop binding to CD4.

MATERIALS AND METHODS

Peptide Syntheses and Photoaffinity Assay. Peptide syntheses of the marker analogues of DB3 and photoaffinity experiment used to produce the covalent complex between DB3 and sCD4 are reported elsewhere (*30*).

Covalent Complex Digestion. The covalent complex solution obtained after irradiation of 81 μg of sCD4 (6.98 nmoles) and 30 equiv of $Phe^1(p-N_3)$DB3 was dried under vacuum. The denaturation of the covalent complex has been achieved by treatment with 100 μL of 7 M guanidine solution for 23 min. The separation of the covalent complex from sCD4 has been obtained by HPLC (eluent A, 0.05% TFA in water; eluent B, 0.05% TFA in CH_3CN; column, Vydac C_{18}, 5 μm, 300 Å, 4,6 × 250 mm); gradient, at 0% B for 10 min, 0%-35% B over 35 min, 35−50% B over 60 min, 50−100% B over 1 min, at 100% B for 10 min; flow rate, 1 mL/min; detector, 214 and 280 nm).

The covalent complex DB3-sCD4 (41.1 μg) and 3 μg of endoproteinase Asp-N (Boehringer Mannheim Gmbh Germany) have been dissolved with 41 μL of 50 mM Na_2HPO_4, pH 8.0 at 37 °C for 6 h. The same digestion has been carried out using 29.69 μg of sCD4. At the end of incubation period, the samples were left at 100 °C for 5 min and then lyophilized.

Separation of the endoproteinase Asp-N digest has been carried out on a C_{18} reversed-phase column (218TP52 Vydac, 2.1 × 250 mm, 5μm) using the following conditions: eluent A, 0.1% TFA in water; eluent B, 0.07% TFA in CH_3CN; gradient, 5−70%B over 90 min, flow rate, 0.2 mL/min; temperature, 37 °C; detector, 220 nm. The peptide identity was confirmed by MALDI analysis of each HPLC fraction after trypsin hydrolysis (substrate/enzyme ratio 50:1 (w/w) in 0.4% ammonium bicarbonate, pH 8.5, at 37 °C for 4 h).

MALDI/MS. MALDI mass spectra have been acquired using a Voyager DE-Pro (Perseptive Biosystem, Framingham, MS) time-of-flight instrument.

All the spectra have been obtained both in linear and reflecton mode and in positive ion mode. The accelerating voltage was 20 kV, the grid voltage was 85%, and the guide wire voltage was 0.02%.

For the analysis of each HPLC fraction, 1 μL of a matrix solution, prepared by dissolving α-cyano-4-hydroxycinnamic acid (10 μg/μL) in a mixture of 0.1% aqueous TFA/acetonitrile 1/1 (v/v), has been loaded on a stainless steel probe, then dried, and finally added with peptide sample dissolved in 0.1% aqueous TFA (about 5 pmoles/μL).

RESULTS

Peptide syntheses of the marker analogues of DB3 and photoaffinity experiment used to produce the covalent complex between DB3 (sequence YNKRKRIHIGPGRAFYTTKNIIG) and sCD4 are reported elsewhere (*30*).

The HPLC separation of the endoproteinase Asp-N digest of the covalent complex CD4−DB3 is reported in Figure 1. Fragments have been collected and analyzed by MALDI mass spectrometry, and their identity has been defined on

Table 1: Identification by MALDI/MS of Native and Modified CD4 Peptides from the Endoproteinase Asp-N Digest of CD4 Incubated with DB3

time (min)[a]	hplc fraction[b]	expected mass[c] (Da)	peptide sequence[d,e]	measured mass[f] (Da)
28.05	4	1044.6	235−243	1045.3
30.02	6	1323.6	153−164	1324.3
		1395.6	339−351	1395.4
30.86	7	1642.9	230−243	1643.7
32.33	8	1210.3	153−163	1210.4
35.04	10	1721.0	13−27	1721.8
		1830.0	195−211	1830.9
35.53	11	2236.1	13−31	2234.5
		2596.3	66−87	2595.5
37.83	12	1742.0	94−109	1742.1
		2200.4	265−285	2201.3
		2236.1	13−31	2235.3
		2596.3	66−87	2595.1
38.90	13	918.5	56−62	919.1
40.24	15	1270.8	1−12	1270.6
44.28	16	2703.5	DB3-(N_2)	2704.3
42.51	17	1390.6	92−104	1390.1
46.66	18	5458.2	DB3 dimer	5457.6
		1398.5	218−229	1399.0
49.65	19	1741.0	63−77	1741.1
50.13	20	1741.0	63−77	1741.1
		4443.1	63−77+DB3	4442.6
51.31	21	5074.7	105−152	5075.6
		5760.5	10−52 + 80−87[g]	5760.7
55.78	23	1884.4	73−88	1883.3
60.82	26	2095.2	173−191	2095.9
62.63	27	1944.0	63−79	1945.3
		1757.0	285−299	1757.9
		1627.1	64−77	1627.8
		5888.7	53−79 + DB3	5887.8
63.85	28	1757.0	285−299	1757.9
65.33	30	6957.1	53−88 + DB3	6958.9
		6212.7	64−93 + DB3	6213.7
		8241.4	13−84	8243.0
		8621.7	20−93	8623.1
78.19	33	2568.9	212−232	2570.7
		2599.3	87−109	2599.9

[a] Time (min) refers to the retention time of the peaks of the chromatogram shown in Figure 1. [b] Numbers refers to the peaks of the chromatogram shown in Figure 1. [c] Mass calculated on the basis of the sequence of the protein CD4 (Swiss-ProtP.A.N.: P01730). [d] Numbers indicate the sequence of the extreme of each peptide. [e] The numbering in sCD4 used excludes the signal peptide. [f] Average molecular mass expressed in Da. [g] Disulfide bridge.

the basis of the CD4 amino acid sequence comparison and enzyme specificity (Table 1). Six mass signals are not ascribed to sCD4 digestion. Two of them, *m*/*z* 2704.3 (fraction 16) and 5457.6 (fraction 18), correspond to DB3 loss of N_2 and to a DB3 dimeric form, respectively. Mass signals, 4442.6 (fraction 20), 5887.8 (fraction 27), and 6958.9 and 6213.7 (fraction 30) are due to peptides of CD4 modified by covalent addition of DB3, as indicated in Table 1. Peptide identity has been confirmed by MALDI/MS fragmentation experiments using the PSD technique or, when necessary because of the high mass of the peptides, performing tryptic mapping by MALDI/MS of the HPLC collected fraction. This was the case of the signal at *m*/*z* 4442.6, present in the fraction 20 spectrum, corresponding to CD4 fragment 63−77 covalently bound to DB3 (Figure 2). Peptide identity has been confirmed by trypsin subdigestion, followed by MALDI analysis of the digestion products. Identity of the fragments obtained by trypsin digestion of fraction 20 is reported in Table 2. The other fragments, observed in the spectrum of the tryptic digest (Figure 3), are due to the hydrolysis of two additional peptides contained in the HPLC fraction 20. These peptides represent longer fragments of CD4, containing the sequence 63−77 and linked to DB3. These results allowed us to locate the binding site of DB3 on the CD4 molecule in the region 63−77 (DQGNFPLIIKNLKIE).

In addition, the subdigestion experiment has shown that the amino acid of fragment 63−77 directly bound to the first DB3 amino acid lies within the sequence 73−75 (NLK). The endoproteinase Asp-N digest of the complex includes DB3-containing fragments of CD4 with sequence extended to position 93.

DISCUSSION

Photoaffinity experiments have demonstrated that DB3 binds the region 63−77 of CD4. In particular the minimal DB3-bound CD4 fragment lies within the sequence 73−75. Considering that the photoaffinity marker was introduced in the first N-terminal residue of DB3 and that the amino acid substitutions that affect the biological activity are relative to the central residues of the 23-mer peptide, we suggest that the CD4 region involved in the interaction with DB3 spans from residues 73−75 to the negatively charged Ig-CDR3-like region (81−96) (Figure 4). This interpretation is in agreement with the finding, in the endoproteinase Asp-N digest of the complex, of DB3-containing fragments of CD4 with sequence extended to position 93 (peptides 53−79, 53−88, and 64−93). These fragments are not present in the form unbound to DB3 (see Table 1) and were absent or very weak in the digest of native CD4 (unpublished data). This may indicate that the region of CD4 sequence binds very tightly to DB3, preventing hydrolysis by endoproteinase Asp-N. Furthermore, this interpretation is also in agreement with the Phe^{67} inaccessibility due to a practically null degree of exposure (*5*), and it is supported by the detection in Asp-N digest of a quantitatively less relevant fragment in which the peptide DB3 is bound to the sequence 85−90 of CD4 (unpublished data). The results of the present photoaffinity experiment suggest the existence of an interaction between gp120 V3 loop and the 63−93 sequence of CD4 (Figure 4).

The role in the gp120−CD4 interaction of the CD4 63−93 region is suggested by several observations: (i) Over 200 mutant CD4 recombinants have been constructed and tested for gp120 affinity (*31*−*33*). Eighty out of the 98 residues in D1 have been analyzed and only 19 positions have an impact on gp120 binding without inducing apparent global conformational changes. Replacements with Ala at Glu^{77}, Asp^{81}, and Glu^{85} fall outside of the 41−59 region, corresponding to the gp120 principal binding site. These three residues are comprised in the CD4 region involved in DB3 binding. (ii) Two major groups of D1-binding mAbs have been described. The epitope of one group, typified by Leu3a (OKT4A, OKT4D), maps the CDR2-like loop (Figure 4) and usually includes positions of the CDR1-like loop. These antibodies block gp120 binding. The epitope of the second group, typified by monoclonal 13B8 (*34*) (L71, MT151, VIT4), includes the CDR3-like loop. These antibodies inhibit viral infection and cell fusion, but are unable to efficiently block gp120 binding (*5*, *35*, *36*). Apparently, these mAbs interact with the CD4 moiety of the CD4-gp120 complex and prevent a post-binding event necessary for membrane fusion. (iii) After the initial binding, entry of virus into cell occurs through fusion. In addition, HIV-infected $CD4^+$ cells fuse with uninfected $CD4^+$ cells to form syncytia, a process

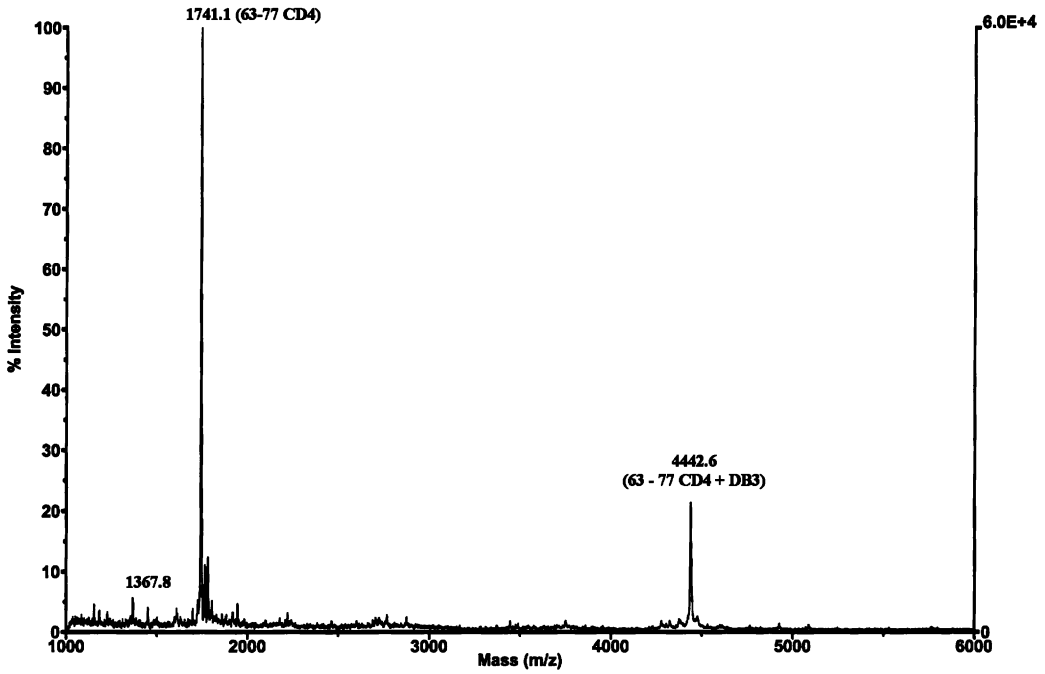

FIGURE 2: MALDI/MS spectrum of fraction 20 obtained from HPLC separation of the endoproteinase Asp-N digest of the covalent complex CD4−DB3. The peptide fraction has been collected from the HPLC separation (Figure 1), under the conditions described in Methods, concentrated to 10 μL, and then analyzed by MALDI/MS.

Table 2: Analysis by MALDI/MS of the Tryptic Digest of the Peptides Contained in Fraction 20 (eluted at 51.31 min, Figure 1) from the Endoproteinase Asp-N Digest of the CD4−DB3 Complex

measured mass[a]	expected mass[b]	peptide sequence[c]
1145.1	1145.3	(63−72) CD4
1235.3	1235.8	(73−75) CD4 + (1−6) DB3
1963.4	1965.2	(73−75) + (1−13) DB3
749.9	749.4	(7−13) DB3
906.1	905.6	(6−13) DB3

[a] Average molecular mass expressed in Da. [b] Mass calculated on the basis of the sequence of the peptide 63−77 of the protein CD4 (Swiss-ProtP.A.N.: P01730) covalently bound to DB3. [c] Numbers indicate the sequence of the extreme of each peptide.

mediated by interactions between HIV envelope proteins expressed on the infected cell surface and CD4 on the uninfected cell. Chimpanzee CD4, which binds to HIV but does not support syncytium formation, has Gly replacing Glu at position 87. This replacement in human CD4 abolishes syncytium formation while preserving gp120 binding (*37*). Mutagenesis studies have shown that residues Asp^{88} and Gln^{89} of the CDR3-like loop are also implicated in this fusion process. These residues lie at the β-turn tip of the CDR3-like loop in D1 and are part of a patch of negative potential on the otherwise mostly positive D1/D2 surface. The CDR3-like loop is spatially separated from the CDR2-like loop implicated in high-affinity binding to gp120. Accordingly, mAbs of the L71 family block syncytium formation although permitting gp120 binding to CD4. Consequently, it was proposed that the initial interaction with the Ig-CDR2-like region could be a prerequisite step for HIV infection on $CD4^+$ cells but may not be sufficient for the subsequent events leading to envelope-CD4-mediated fusion of viral and cellular membranes. A second region, CDR3-like loop, is probably required for events that occur downstream of the initial binding step. Inhibition of this site by blocking with CD4 mAbs of L71 family causes only limited interference with gp120 binding to CD4 but strongly blocks HIV infection of $CD4^+$ cells and fusion between HIV-infected and uninfected cells. In addition it was demonstrated that a synthetic aromatically modified exocyclic form, derived from the CDR3-like loop (residues 82−89) region of CD4 D1 domain, specifically inhibits binding of recombinant gp120 to both recombinant soluble CD4 and $CD4^+$ Jurkat cells and blocks syncytium formation and virus particle production caused by HIV infection *(38)*. Both mAbs of L71 family and molecules derived from CDR3-like region block fusion process by the occupation of CDR3 site or creating a disabled CD4 heterodimer. Probably these molecules are able to interfere in the direct interaction of Ig-CDR3-like region with the envelope protein gp120. Considering the results reported in this paper, we suggest that the V3 loop of viral envelope glycoprotein gp120 could be the counterpart of Ig-CDR3-like region in the binding between gp120 and CD4. Probably DB3 is able to promote syncytium formation miming the role of the entire V3 loop in CD4 binding. The DB3/CD4 interaction is affected by positively charged residues of the peptide. In particular, the substitution of Lys or Arg residues with Asn causes a decrease in the binding of DB3 point mutated analogues to sCD4 *(28)*. In detail, the substitution $Lys^3 \rightarrow$ Asn produces a binding decrease of 15%, whereas $Arg^6 \rightarrow$ Asn or $Lys^{19} \rightarrow$ Asn causes a 50% binding decrease. In addition, we have synthesized a peptide named DB3M in which the N-terminal and C-terminal basic residues of the DB3 sequence were deleted (7−18 sequence of DB3). The sCD4 binding of DB3M is only the 10% of DB3 binding. Consequently we have shown the importance of basic amino acids of DB3 for efficient binding to CD4. Nevertheless, the idea of a mere electrostatic interaction between DB3 and Ig-CDR3-like region is misleading. In fact, the substitution of aromatic residues with hydrophobic aliphatic residues, such as Ile ($Phe^{15} \rightarrow$ Ile and $Tyr^{16} \rightarrow$ Ile analogues), causes

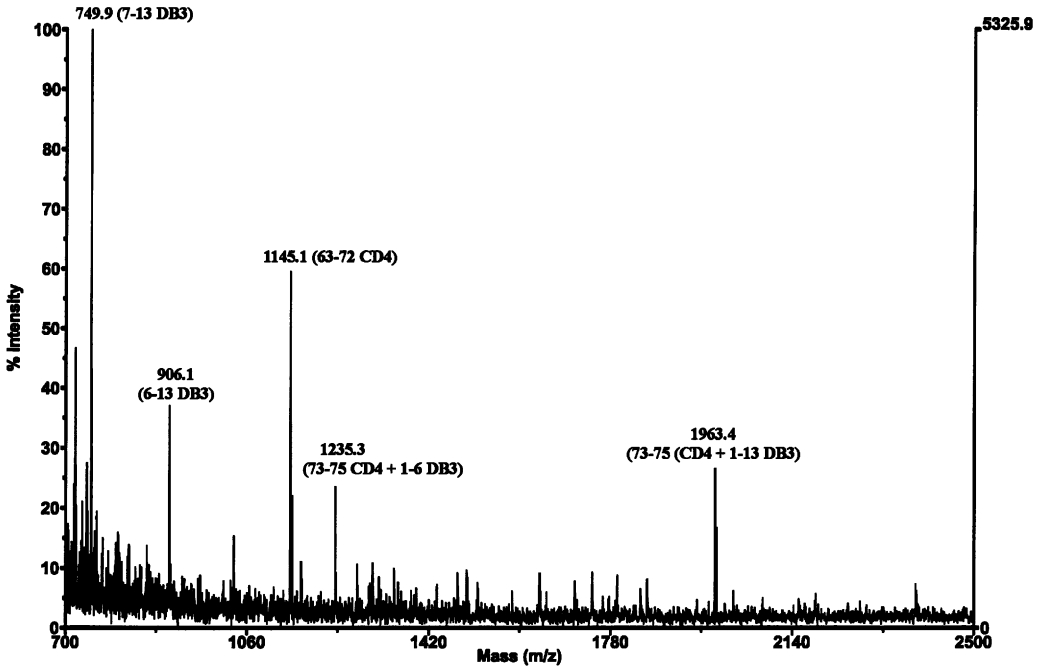

FIGURE 3: MALDI/MS spectrum of the tryptic digest of fraction 20 obtained from the endoproteinase Asp-N digest of the covalent complex CD4−DB3. Fraction 20 from the HPLC separation reported in Figure 1 has been subdigested with trypsin and the resulting pepide mixture has been analyzed by MALDI/MS

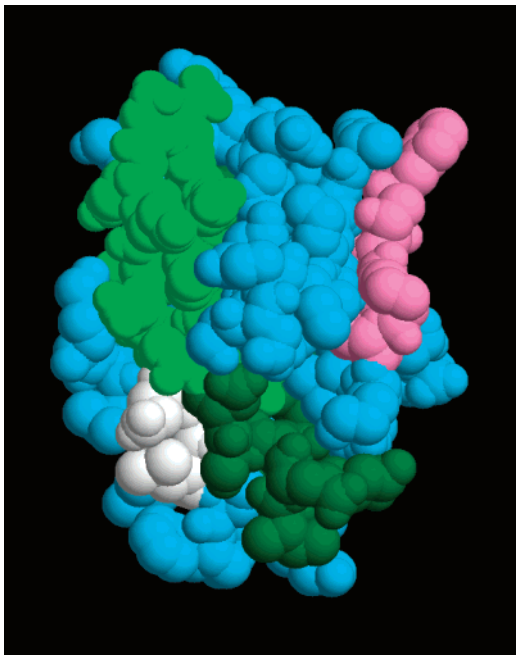

FIGURE 4: Space-filling model of the D1 atomic structure. The background atoms of D1 (residues 1−98) are colored in blue. Residues 42−49, colored pink, represent CDR2-like region. Residues 63−93, colored green, represent the binding region of DB3. Residues colored white represent the CDR3-like region. The overlapping sequence between the DB3 binding site (dark green) and the CDR3-like region (white) is colored in light green.

a remarkable decrease, 80% and 90%, respectively, of sCD4 binding to the 20% or 30% in comparison with DB3. Interestingly the binding activity of different analogues parallels their enhancing effect on HIV infection.

Despite these evidences, the X-ray structure of gp120−CD4 complexes (*9*) shows that it is impossible for a single gp120 molecule to bind to CD4 with both the gp120 principal binding site and the V3 loop; in fact, after gp120 binding to Ig-CDR2-like loop, the CDR3 loop is almost 50 Å away from the V3 base and is on the opposite face of CD4. Consequently it is impossible for V3 loop to reach it, even if the loop was a totally extended structure. On the other side, considering that the gp120 molecule is present on the viral surface in a trimeric form, it can be supposed that the binding among CD4-gp120 and HIV-1 coreceptor could involve more gp120 units.

In addition, the DB3 might be involved in the mechanism of nuclear translocation of NF-kB. In fact, HIV-binding to CD4 also triggers signals that lead to nuclear translocation of NF-kB and that are important for the productive infection process (*34*). Indeed, it has been suggested that basal transcription is regulated by NF-kB protein whereas "late" transcription is regulated by Tat and Sp-1. In this cascade of signals, both the CD4 cytoplasmic tail and the immunoglobulin CDR3-like loop are required for signal transduction: the substitution of negatively charged residues for positively charged residues within the CDR3-like loop inhibits NF-kB translocation.

In conclusion, considering the results of the present photoaffinity experiment, we suggest that the V3 loop of viral envelope glycoprotein gp120 and the sequence that precedes and includes a part of the CDR3-like region of CD4 could be involved in a secondary binding considered very important for syncytium formation and for triggering CD4 signaling transduction.

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