The principal neutralizing determinant of HIV-1 located in V3 of gp120 forms a 12-residue loop by internal hydrophobic interactions

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Abstract The interactions of the peptide RP135a (RKSIRIQRGPGRAFVT), corresponding to residues 311–326 of gp120 of HIV-1_{IIIB}, with the anti-gp120 HIV-1_{IIIB} neutralizing antibody 0.5β were studied by NMR. The NOESY difference spectra measured using specifically deuterated derivatives of the peptide show exclusively the interactions of the deuterated residues both within the bound peptide and with the Fab fragment of the antibody. These measurements reveal hydrophobic interactions within the bound peptide between Ile-4, Ile-6 and Val-15 that create a 12-residue loop with these residues at the base and the conserved GPGR sequence at its top.

Key words: NMR, ^{1}H; Antibody; Antigen conformation; HIV-1; gp120

1. Introduction

The principal neutralizing determinant (PND) of the human immunodeficiency virus type 1 (HIV-1) has been mapped to a 24-amino acid-long sequence (NNTRKSIRIQRGPGRAFVTIGKIG) corresponding to residues 308–331 of the virus envelope glycoprotein, gp120 [1–3]. This sequence, denoted RP135, is part of the third hypervariable region (V3) which forms a disulfide cross-linked 36-residue loop [4]. RP135 and its shorter derivatives were found to elicit HIV neutralizing antibodies, thus demonstrating their potential use in a peptide based vaccine [2]. The 0.5β antibody is a very potent strain-specific virus neutralizing antibody which was raised against gp120 of HIV-1_{IIIB} [5]. Passive immunization with a chimeric antibody which contains the intact variable regions of 0.5β was found to protect chimpanzees from HIV infection [6]. The antibody 0.5β binds gp120 and the peptide RP135 with comparable affinities (2×10^8 and 7×10^7 M^{-1}, respectively) [7].

The structure of gp120 is not known and there is no structural information on anti-gp120 antibodies and their complexes with their corresponding antigens. To study the conformation of PND peptides as they are recognized by anti-gp120 virus neutralizing antibodies directed against the V3 loop, it is possible to use NMR and especially two-dimensional NOE spectroscopy (NOESY). NOESY measurements detect interactions between hydrogen nuclei (protons), that are separated by up to 5 Å [8,9]. In the two-dimensional spectrum these interactions are manifested by cross-peaks that are characterized by the resonance frequencies of the two interacting nuclei. As shown in the spectra of the 0.5β Fab fragment (50 kDa) presented in Fig. 1A and B, NOESY spectra of large proteins suffer from lack of resolution due to the large number of protons in the molecule and the broadening of their individual resonances that occurs as the molecular weight of the protein increases. Moreover, techniques that are used to assign resonances to the corresponding protein protons (sequential assignment) are currently not applicable for proteins of the size of the Fab fragment of the antibody [9,10]. Transferred NOE difference spectroscopy has been used to study antibody–peptide complexes and to obtain simplified spectra that show exclusively cross-peaks due to antibody–peptide interactions and interactions within the bound peptide [11]. Unfortunately, this technique is not applicable for the 0.5β–RP135 complex due to its very slow dissociation rate [12]. In such case difference spectra calculations using deuterated peptides could be applicable, as was demonstrated in studies of pepsin interactions with a tri-peptide inhibitor [13].

2. Materials and methods

2.1. Peptides and Fab

The peptide RP135a (RKSIRIQRGPGRAFVT) was synthesized and purified as described previously [12]. Perdeuterated L-amino acids (isoleucine, proline, alanine and valine) and L-phenyl-d_5-alanine were obtained from MSD Isotopes, Canada. Minimum isotopic purity of the deuterated amino acids was 98% ^{2}H. For peptide synthesis all deuterated amino acids were protected by *t*-butyloxycarbonyl group (t-boc). Protected deuterated amino acids were synthesized and purified by Oz Chemicals, Israel. Six derivatives of RP135a were prepared by specific deuteration at a single position (Ile-4, Ile-6, Pro-10, Ala-13, Phe-14 or Val-15). Preparation and purification of the Fab fragment was described previously [12].

2.2. NMR spectroscopy

The two samples used for each difference spectrum measurement contained the same concentration of the Fab–RP135a complex, using Fab from the same cleavage batch. Concentration of the complex for all difference spectra was 1–1.4 mM in 10 mM phosphate-buffered D_2O pH 7.15 (meter reading) containing 0.05% NaN_3. NOESY spectra were measured at 315 K on a Bruker AM500 with a 70 ms mixing period. The residual HOD signal was suppressed by gated irradiation. A total of 256 transients were acquired in the t_1 domain with 80×2K free induction decays acquired for each t_1 value. To obviate the use of a phase correction in the F_1 dimension, the sampling delay, d0, was set to $d0 = [IN0/2] - [2PW/\pi]$ where IN0 is the dwell time in the F_1 dimension and PW is the length of the 90° pulse (Bax et al., 1991). The data was zero filled in the F_1 dimension, multiplied in both dimensions by a square sine bell window shifted by 60° and then Fourier transformed in both dimensions. To yield minimal distortions in the difference spectrum baseline, the phases of the two spectra (NOESY of Fab with unlabeled peptide and NOESY of Fab with specifically deuterated peptide) were very carefully adjusted prior to subtraction. By matching pairs of rows from the two spectra, a difference factor is chosen (usually 1.0 or very close to 2^n, where n is a scaling factor between the spectra) to minimize the number of both positive and negative cross-peaks and to obtain a flat baseline Typically, a baseline correction is further applied around the HDO resonance of the difference spectrum.

To examine the effect of the mixing time on the signal-to-noise ratio

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in the NOESY and the difference spectrum we measured the NOESY spectra of Fab complex with unlabeled peptide and with a peptide in which Val-15 was deuterated at two different mixing times: 40 ms and 70 ms. The signal-to-noise ratios in the NOESY spectrum (of the resolved cross-peaks of Ile-4) and in the NOESY difference spectrum (Val-15 cross-peaks) were 30% higher with 70 ms mixing time in comparison with the spectra measured using 40 ms mixing. Because of the considerable improvement in the signal-to-noise ratio all other NOESY spectra were recorded with 70 ms mixing. The limited number of observed cross-peaks, and the observation that within a given peptide residue only some of the protons give rise to NOE cross-peaks with Fab protons (as shown for Ile-4 in Fig. 1D), and that these cross-peaks vary in their intensity, indicate that spin diffusion is rather limited. Perhaps the higher temperature, and the relatively low concentration used (1.2 mM of Fab) helped to decrease the sample viscosity and subsequently the rotational correlation time of the Fab molecule, thus decreasing spin diffusion effects. Moreover, the signal-to-noise in the difference spectra does not enable the observation of weak cross-peaks that could potentially arise from spin-diffusion.

3. Results

To study 0.5β interactions with the PND of HIV-1_{IIIB} we used the peptide RP135a (RKSIRIQRGPGRAFVT) which contains the epitope recognized by the antibody [12]. The difference between the one-dimensional (1D) ^{1}H NMR spectrum of the Fab complex with the unlabeled peptide and that of the Fab complex with a specifically deuterated peptide shows the resonances of the labeled residue in the bound peptide (data not shown). The difference between the NOESY spectrum of the Fab complex with the unlabeled peptide and the NOESY spectrum of the Fab complex with a specifically deuterated peptide shows the NOE cross-peaks of the labeled residue. The NOESY difference spectrum and the 1D difference spectrum measured for each specifically deuterated peptide are used together to assign the resonances of the labeled residue to the correspond-

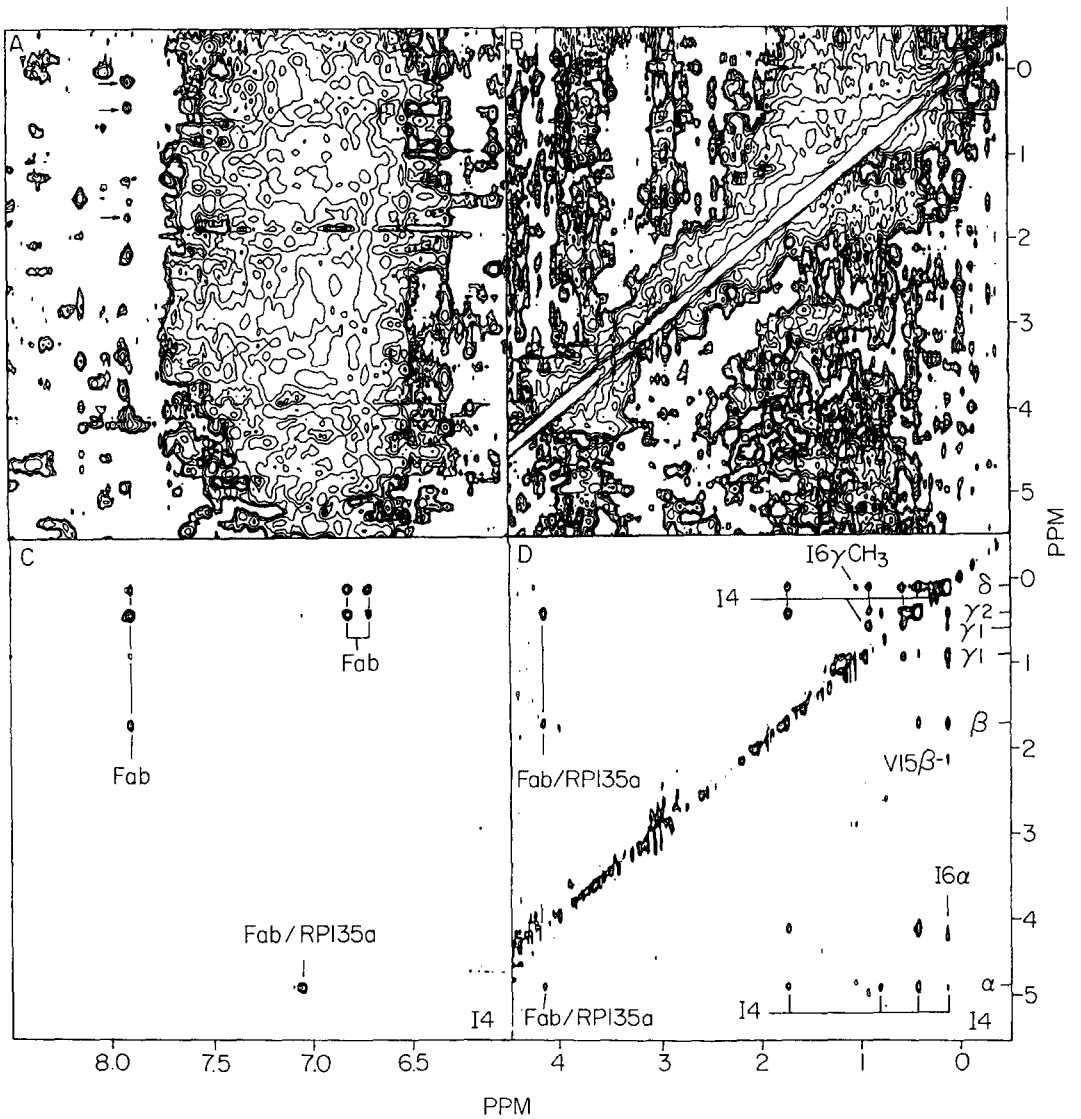

Fig. 1. A comparison between regions of the NOESY spectrum of the 0.5β Fab complex with the peptide RP135a (RKSIRIQRGPGRAFVT) corresponding to residues 311–326 of gp120 of HIV-1_{IIIB} (Fig. 1A and B) and the difference between this spectrum and the spectrum of the complex in which Ile-4 of the peptide was deuterated (Fig. 1C and 1D). The difference spectrum shows the NOE cross-peaks of the deuterated residue. The location of Ile-4 resonances are marked on the y-axis. The arrows in Fig. 1A mark the locations of Ile-4 cross-peaks observed in the difference spectrum (Fig. 1C). Cross-peaks due to intra-residue interactions are marked by I4, interactions within the bound peptide are labeled with the the symbol of the proton interacting with Ile-4 i.e. V15β, and cross-peaks that could be due to either RP135a/Fab interactions or interactions within RP135a are marked as such (i.e. Fab/ RP135a).

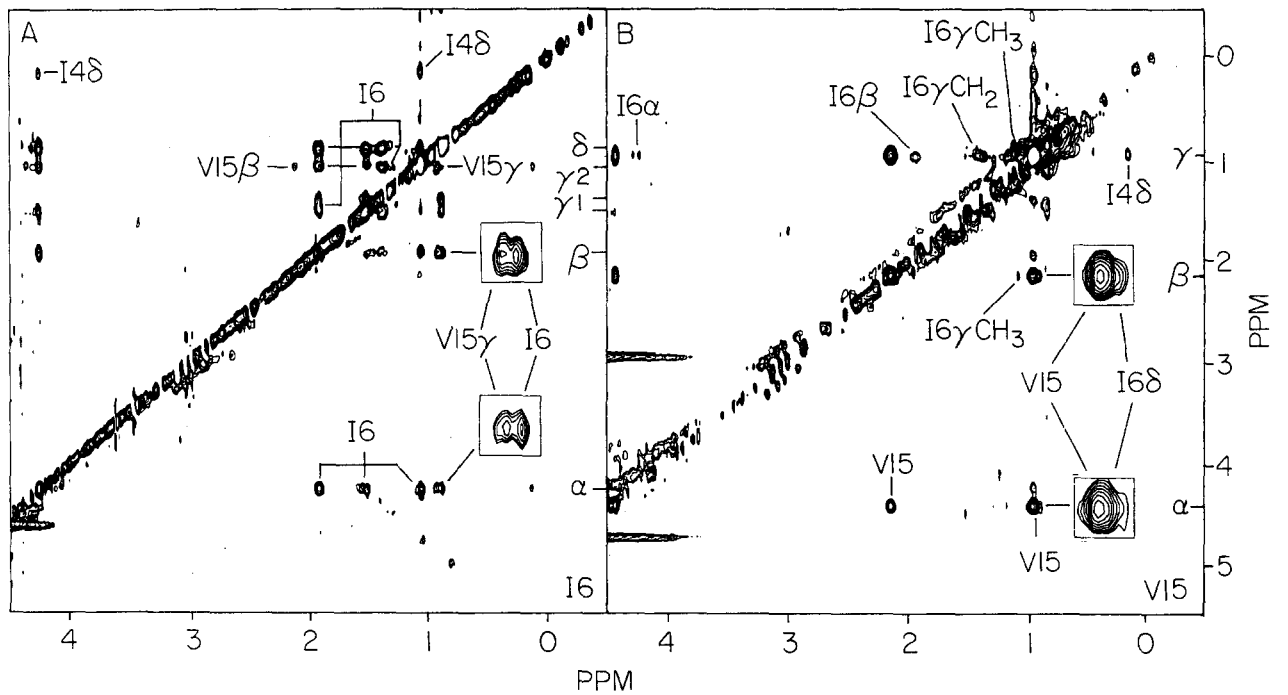

Fig. 2. The difference between the NOESY spectrum of 0.5β Fab complex with the peptide RP135a (RKSIRIQRGPGRAFVT) and the spectrum of the Fab complex with a peptide molecule in which a specific peptide residue has been deuterated: (A) Ile-6 and (B) Val-15. The difference spectrum shows the NOE cross-peaks of the deuterated residue in the aliphatic region of the spectrum. The locations of the resonances of the deuterated residues are marked on the y-axis and cross-peaks are labeled as in Fig. 1. The inserts give a blow-up of the marked cross-peaks.

ing protons and to discern NOESY cross-peaks due to intra-residue interactions from cross-peaks due to inter-residue interactions. The partial assignment of the bound peptide resonances is summarized in Table 1.

The NOESY difference spectrum measured for a Fab complex with a peptide in which Ile-4 was deuterated is shown in Fig. 1C and D. This spectrum exhibits with a remarkable signal-to-noise ratio only NOE cross-peaks of Ile-4; all other cross-peaks not involving Ile-4 are cancelled out. All cross-peaks in Fig. 1C, except one, were assigned to Ile-4 interactions with aromatic protons of the Fab. On the basis of its chemical shifts, the cross-peak at (4.79, 7.02 ppm) could be assigned to interaction with the Fab or with $C_\varepsilon H$ of Phe-14 of RP135a. Fig. 1D reveals interactions of Ile-4 δCH_3 with three peptide protons: Ile-6 γCH_3, Ile-6 αH and Val-15 βH. Two additional cross-peaks in Fig. 1D (0.40, 4.14 and 1.70, 4.14 ppm) are due to Ile-4 interactions with Fab protons or yet unassigned peptide protons.

Table 1
Resonance assignments of RP135a bound to the 0.5β antibody

Residue	αH	βH	others
Ile-4	4.79	1.71	γCH_2: 0.9, 0.57; γCH_3: 0.39; δCH_3: 0.09
Ile-6	4.24	1.89	γCH_2: 1.49, 1.37; γCH_3: 1.03; δCH_3: 0.86
Pro-10	4.19		δCH_2: 3.24, 3.02
Ala-13	3.97	1.3	
Phe-14	5.01	2.95, 2.73	$C_\delta H$: 7.14; $C_\varepsilon H$: 7.02
Val-15	4.42	2.12	γCH_3: 0.90

Chemical shifts are given in ppm relative to 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt.

According to the difference spectrum presented in Fig. 2A, γCH_3 of Ile-6 interacts with three peptide protons: Ile-4 δCH_3, Val-15 γCH_3 and Val-15 βH. Intra-peptide interactions are observed also between Ile-6 βH and Val-15 γCH_3 and between Ile-6 αH and both Ile-4 δH and Val-15 γCH_3. According to the difference spectrum presented in Fig. 2B Val-15 protons are involved in a number of long range interactions within the bound peptide: (a) Val-15 γCH3 interacts with the following peptide protons: Ile-4 δCH_3, Ile-6 αH, Ile-6 βH, Ile-6 γCH_3 and one of the Ile-6 γCH_2 protons; (b) Val-15 βH interacts with Ile-6 δCH_3 and Ile-6 γCH_3; and (c) Val-15 αH interacts with Ile-6 δCH_3. The observation that most of the interactions between Ile-4, Ile-6 and Val-15 appear twice in the presented spectra confirms our assignment; i.e. an interaction between Val-15 and Ile-6 appears once in the difference spectrum measured for a Fab complex with a peptide in which Val-15 is deuterated and once in a difference spectrum measured for Fab complex with RP135a in which Ile-6 is deuterated.

Ile-6, Pro-10 and Phe-14 were found to interact with aromatic protons of the Fab (data not shown). The aromatic protons of Phe-14 and the methyl protons of Ala-13 were found to interact with non-aromatic protons of the Fab or yet unidentified protons of RP135a (data not shown). No additional interactions within the bound peptide could be assigned.

4. Discussion

The extensive network of hydrophobic interactions in the bound peptide between Ile-4, Ile-6 and Val-15 indicates that the sequence IRIQRGPGRAFV, corresponding to residues 314–325 of HIV-1_{IIIB} gp120, forms a 12 residue loop with Ile-4, Ile-6 and Val-15 at the base and the conserved GPGR sequence at

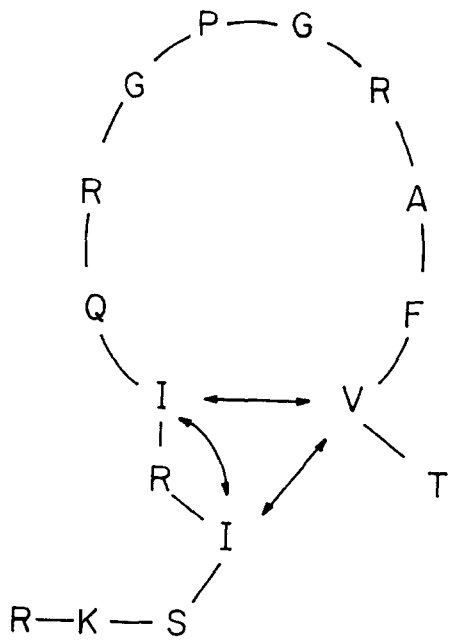

Fig. 3. A schematic illustration of the 12-residue loop formed within the V3 peptide RP135a bound to the anti-gp120 HIV-neutralizing antibody 0.5β. The arrows mark the observed interactions.

the top as schematically illustrated in Fig. 3. This 12-residue loop formed by non-covalent interactions is part of the larger 36-residue V3 loop formed by a disulfide bridge between Cys-303 and Cys-338 of gp120 [4]. The hydrophobic interactions observed in RP135a bound to the anti-gp120 antibody 0.5β were not observed in free RP135 [15] nor were they observed in the crystal structure of complexes of antibodies raised against PND peptides of the MN strain [16,17]. The PND peptide of the MN strain (RP142) shares 52% identity with RP135 [15]. The two anti-MN peptide antibodies that were studied by X-ray crystallography recognize only 7-residue epitopes, considerably shorter than the 14–16 residue epitope recognized by 0.5β [12]. Anti-gp120 antibodies have not been studied before neither by X-ray crystallography nor by NMR spectroscopy. Our study demonstrates the benefits of using anti-gp120 antibodies that recognize a large epitope to obtain the global conformation of the V3 loop. As the 0.5β antibody was raised against gp120, it is expected that the conformation of the flexible PND peptide bound to the antibody is similar to the conformation of the corresponding part of gp120 when bound to the same antibody. In this case the more rigid antibody raised against gp120 will serve as a template for the folding of the peptide. Peptides corresponding in sequence to segments of the V3 loop have been used to elicit HIV-1 neutralizing antibodies [2]. In view of our findings, it is possible that chemically constrained synthetic peptides that contain the 12-residue loop will be more efficient than flexible peptides in eliciting highly cross-reactive and potent HIV-1 neutralizing antibodies.

Ile-4 and Ile-6 of RP135a correspond to Ile-314 and Ile-316 of gp120, which are conserved in 94% and 82% of gp120 sequences, respectively [18]. In only 1.6% and 3.2% of gp120 sequences do polar residues appear in positions 314 and 316, respectively. Val-15 of RP135a corresponds to Val-325 of gp120. Although valine is not the most common residue in position 325, 94% of gp120 sequences have a hydrophobic residue in this position. Residue 324 in gp120 is Phe in 73% and hydrophobic in 98.7% of gp120 sequences. This sequence comparison indicates that in the vast majority of HIV isolates, gp120 has hydrophobic residues that could potentially participate in hydrophobic interactions that stabilize a loop similar to that observed for RP135a. Since the peptide RP135a of the HIV-1_{IIIB} isolate contains an insertion of two residues (Gln-7 and Arg-8) that appear in only 4% of gp120 sequences [18], this loop may be 2-residues shorter in gp120 of other strains. The NOESY difference spectroscopy presented in this study could be implemented for comparative studies of complexes of anti-gp120 antibodies recognizing the PND of other HIV strains to find out whether the loop found in this study for the HIV-1_{IIIB} strain is indeed widespread among HIV isolates.

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