Journal of Peptide Science, Vol. 3, 383–390 (1997)

The Design of a Specific Ligand of HIV gp120

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Received 9 April 1996
Accepted 18 July 1996

Abstract: The crystal structure of CD4 suggested that the C/G^{38} and C/L^{44} replacements with the consequent cystine bridge formation are compatible with the native structure of that molecular moiety. As the NQGSF sequence, corresponding to the 39–43 fragment of human CD4 protein, was found to be involved in the HIV gp120 interaction, it has been synthesized in a cyclic form by adding two cysteine residues at the amino and carboxy termini. ^{1}H-NMR studies show that the predominant solution conformation of cyclo-[CNQGSFC] is a type II β-turn centred on the NQGS segment. Structural and dynamic properties of the peptide are also analysed in relation to the *in vitro* activity. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

J. Pep. Sci. 3: 383–390

No. of Figures: 7. No. of Tables: 1. No. of References: 33.

Keywords: CD4; gp120; NMR; peptides; structure

INTRODUCTION

During the early stages of the HIV infection, the viral glycoprotein gp 120 specifically binds to the CD4 antigen expressed on the surface of the target cells [1–4]. This recognition is followed by a number of steps involving other cell surface molecules, leading to the virus entry through an envelope-mediated fusion with the host plasmatic membrane [5]. The interaction between CD4 and gp120 is also responsible for the cytopathic effects typically observed *in vitro* on infected CD4$^+$ lymphocytes: the heterologous expression of the envelope glycoprotein following the virus infection indeed stimulates the formation of giant multinucleate syncytia, reproducing the molecular recognition of HIV I particles with their cellular receptor [5, 6].

The CD4 glycoprotein is a member of the superfamily of immunoglobulins. Its first amino-terminal domain D1 shares both sequence and topology similarity with the light chain variable region of antibodies [7–9]. The molecular structure of the amino-terminal half of human CD4, spanning from 1 to 183 residues, results in an all β-type architec-

Abbreviations: CD4, the complementarity-determining antigen; C′ and C″, respectively, the 34–39 and 42–46 regions of CD4; D1, one of the CD4 protein domain; gp120, a HIV capsidic glycoprotein; HIV, human immunodeficiency virus; $J_{\alpha N}$, the ϕ vicinal coupling constant; Mabs, monoclonal antibodies; NOE, nuclear Overhauser effect; RI1, the cyclic heptapeptide CNQGSFC.

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CCC 1075-2617/97/050383–08 $17.50

ture, composed of two β-barrel domains closely packed together. Experimental mapping of the gp120 binding site on the CD4 surface has been obtained during the last few years by the identification of escape mutants unable to recognize the viral glycoprotein efficiently [10–15]. An alternative approach using synthetic peptides was also reported [16–18]. All the reported results suggest a self-consistent framework for the CD4–gp120 interaction, where: (i) the HIV binding site comprises between residues 28 and 60 of the first amino-terminal domain D1 of CD4 and consists of discontinuous segments; (ii) two clusters of amino acids, 40–44 and 51–55, probably define a molecular area more directly involved in the gp120 contacts. The crystallographic structure available for the first amino-terminal domain of CD4 [19–21] has been fundamental in evaluating the effects of site-specific substitutions impairing the gp120 recognition.

As shown by this solid-state structure, fragments 40–44 and 51–55 correspond to exposed surface loops which connect β strands. This feature suggests a possible mechanism for the virus interaction: a double anchoring on the CD4 surface by the latter protruding fingers with the sequences GNQGSF and PKLS respectively, arranged in two β-turns.

In the present study the X-ray data have been used for the rational design of a cyclic peptide reproducing the CD4 38–44 bioactive fragment.

MATERIALS AND METHODS

The linear peptide CNQGSFC was synthesized with a solid-phase automatic synthesizer (Applied Biosystem 430A) using the *t*-Boc amino-acid derivatives. After deprotection of amino- and carboxy- groups, samples were purified by reversed-phase chromatography on a m-Bondapak C18 column. Cyclization of the peptides was obtained by dissolving the lyophilized products in 2 of 3 mM phosphate buffer (pH 8) and stirring for 1 h. Then, once the absence of free sulphydryl groups was established with the Ellman reagent, samples were concentrated and lyophilized. The oxidation process was followed by an HPLC control: the chromatographic profile showed the absence of the peak corresponding to the linear peptide and the appearance of another peak with shorter retention time. Peaks with longer retention times probably corresponded to polymeric products, while short retention time peaks were identified as scavengers and side-products. The oxidized product, corresponding to the main peak, was recovered by lyophilization. Then it was repurified, collected, pooled and lyophilized.

CD measurements, spanning 350 to 200 nM, were carried out using a JASCO JC500 spectropolarimeter. Spectra were recorded at room temperature using a sensitivity value of 1 millidegree/cm and a time constant value of 1 s.

NMR measurements were performed on samples where small quantities of ^2H_2O were added to the water peptide solution to reach a 90% ^1H_2O 10% ^2H_2O v/v ratio. In order to minimize the chemical exchange between peptide labile protons and solvent molecules, a sample pH of 3.5 was adjusted with NaOH and HCl concentrated solutions. Proton NMR spectra were acquired on a Bruker AM 500, equipped with an Aspect 3000 computer. The methyl hydrogen resonance of sodium 3-(trimethylsilyl)propionate was used as the chemical shift internal reference. A two-dimensional TOCSY experiment with a mixing time of 54 ms was carried out to assign the resonances within the spin systems. On the same sample for sequence-specific assignment and detection of dipolar couplings a ROESY experiment [22] was acquired by using a repetitive pulse sequence with a period (β–τ) for mixing, with $\beta = 30°$ and $\tau = 20\,\mu s$ over a time of 240 ms. Both experiments consisted of 512 blocks of 2048 data points; the solvent signal was suppressed using a selective low-power presaturation pulse during the relaxation delay. Two-dimensional spectra were weighted in F2 using a cosine-bell function and zero-filled in both dimensions before the Fourier transform. Scalar coupling constants were measured with a 5% accuracy directly from 1D spectra, acquired with 32K data points using a spectral width of 5208 Hz. Five different monodimensional spectra were collected, ranging in temperature from 300 to 340 K. The chemical shifts of backbone amide protons over this range were used to calculate, by linear least-squares fitting, the dependence of chemical shift on temperature.

Structure refinement and molecular modelling were performed on an Indy Silicon Graphics workstation supporting the Sybyl software (Tripos Associates). A first restrained molecular dynamics calculation was carried out starting from a Type II β-turn conformation. The dynamic run was performed at 500 K for 70 ps, integrating the linear equations every 0.3 ps, using the Kollman force field. A set of ten conformers, corresponding to the lower potential energy points reached during the last 20 ps, were then selected and cooled during a

second dynamic run until 50 K. Final refinement was then performed by 2000 cycles of energy minimization using the conjugate gradient method.

Atomic coordinates of human CD4 were retrieved from the Brookhaven Protein Data Bank (entry ID 3CD4).

RESULTS AND DISCUSSION

The rational design of a gp120 ligand from an active CD4 region was carried out by selecting the NQGSF sequence, involved in the binding with the viral glycoprotein. In the native molecule, this fragment corresponds to the second complementarity determining region (CDR2), as evidenced by structural superimposition with several immunoglobulin variable domains [21]. The conformational state of this protein moiety is characterized by the presence of a β-turn. The synthesis of a linear pentapeptide with the latter sequence does not ensure the production of an efficient gp120 ligand including the active conformation, owing to the high flexibility always exhibited by similar linear molecules.

A much used constitutional constraint is the introduction of two cysteine residues at the peptide amino- and carboxy- termini with the consequent disulphide bridge formation. In our case, this modification may be consistent with the native conformation of residues 30–43 of the protein, as suggested by the atomic distance in the crystal of the carbons between the CD4 G^{38} and L^{44} residues. The latter distance, 6.150 Å, is in fact within the average values found in protein cystine bridges [23].

The synthesized cyclic peptide CNQGSFC, henceforth called RI1, was first investigated by means of CD spectroscopy. The observation of a strong positive band centred at 200 nm confirmed that only a small fraction of disordered structures was present in the conformational equilibrium, as the CD spectrum was the typical one for a peptide in a Type II β-turn conformation [24]. The latter data suggested also that a detailed NMR structural investigation could be practicable for the heptapeptide.

In the NMR study, sequential assignment was achieved from 2D NMR spectra using common procedures [25]: the ^{1}H chemical shifts measured at 300 K are listed in Table 1. The existence of a prevalent order in solution for RI1 is hinted at by the large difference of G^4 α proton chemical shifts. Moreover, a reduced flexibility of RI1 is suggested by the measured $J_{\alpha N}$ values, since, except for the N^2 residue, all the amide protons show coupling constant values relatively far from the average values typical of large conformational equilibria. The overall thermal stability of the molecule can be efficiently probed by the temperature dependence of scalar coupling constants. Figure 1 shows the different behaviour with temperature changes of FC^7–C^1N and QGS moieties of the molecule. The former was in fact strongly weakened at higher temperature, as deduced from the progressive annealing of N^2, F^6 and C^7 coupling constants towards random coil values raising the temperature to 340 K; in the same

Table 1 NMR Parameters Derived from 1 D Spectra of the Cyclic CNQGSFC Heptapeptide

AA	NH	Hα	Hβ	Others		$J_{\alpha N}$ (Hz, 300°)	$\Delta\delta/\Delta T$ (ppb)
C1	8.70	4.40	3.30				
N2	8.95	4.76	3.01	$H_{\gamma 1}$ 7.60		6.34	6.70
			2.94	$H_{\gamma 2}$ 7.45			
Q3	8.39	4.27	2.45	H_{γ} 2.10	$H_{\varepsilon 1}$ 7.56	4.84	8.00
			2.10		$H_{\varepsilon 2}$ 6.91		
G4	8.56	4.20				5.72	9.00
		3.80					
S5	8.03	4.54	3.80			8.02	3.00
F6	8.30	4.79	3.10	2,6H		8.38	8.50
				7.3			
				3.25			
C7	8.27	4.79	3.38			8.25	7.70
			3.10				

Chemical shifts from methyl hydrogen resonance of sodium 3-(trimethylsilyl)propionate; $J_{\alpha N}$ values, expressed in Hz, are the coupling constants of amide protons with the vicinal α protons. $-\Delta\delta/\Delta T$ values are the temperature coefficients of amide proton chemical shifts.

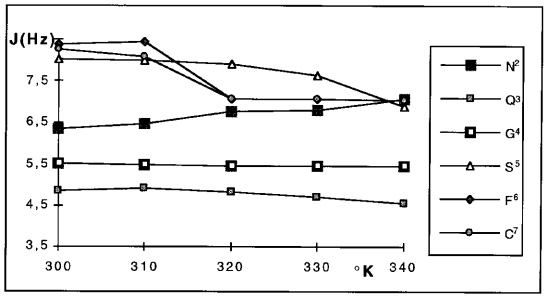

Figure 1 Temperature dependence of vicinal scalar coupling constants measured for the amide protons of the CNQGSFC cyclic heptapeptide.

conditions, Q^3 and G^4 $J_{\alpha N}$ show a remarkable invariance.

Torsion angles ϕ, calculated from $J_{\alpha N}$ [25, 26] at 300 K, are compatible with a type II β-turn centred at the NQGS fragment, since a small value was measured for N^2 and a triplet was observed for G^4. Furthermore, the existence of a β-turn is also supported by the low thermal coefficient of the S^5 NH proton (see Table 1), likely to be involved in an intramolecular hydrogen bond with the N^2 carbonyl group.

Figure 2 shows the RI1 fingerprint region of the TOCSY and ROESY spectra. In the ROESY spectrum no medium or long-range strong dipolar couplings were observed, suggesting that the molecule populates a set of different fluctuating conformations. However, the strong sequential NH_i–NH_{i+1} correlations of N^2, G^4 and S^5 residues (Figure 3) seem to agree with the coupling constant analysis, as the observed NOE connectivity pattern (Figure 4) is compatible with the presence of a type II β-turn structure [25] centred on the NQGS tetrapeptide. As an additional remark, it should be noted that in type II β-turns glycine is often encountered, as in the proposed case, in the $i+2$ position [24].

Model building of RI1 was performed using a combination of experimental data and standard

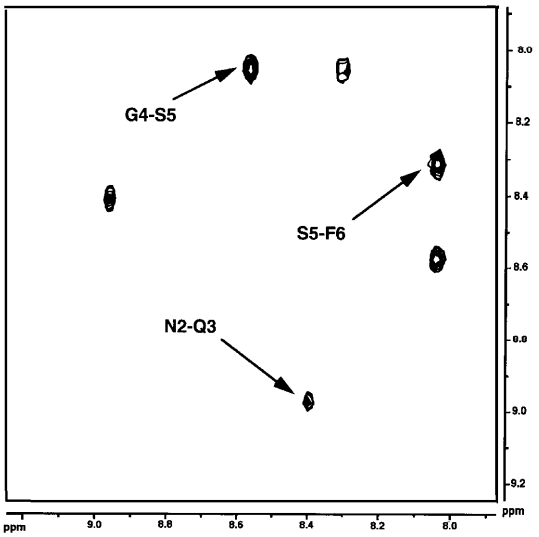

Figure 3 NH–NH dipolar couplings observed in the amide region of the ROESY spectrum of CNQGSFC (only positive levels are shown).

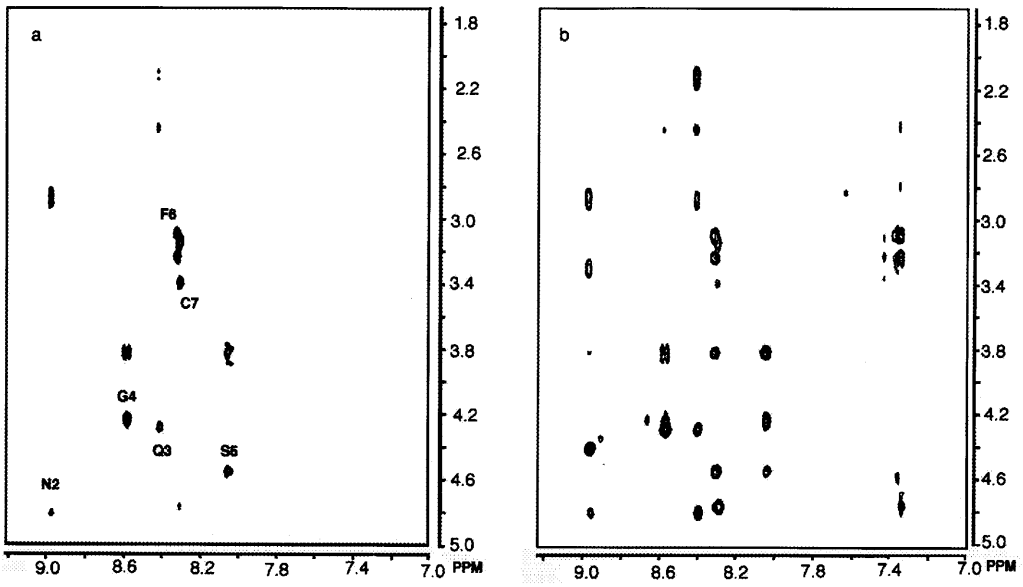

Figure 2 500 MHz ^{1}H-NMR 2D spectra of CNQGSFC in a 90% ^1H_2O 10% ^2H_2O solution: the fingerprint region of (a) TOCSY and (b) ROESY spectra.

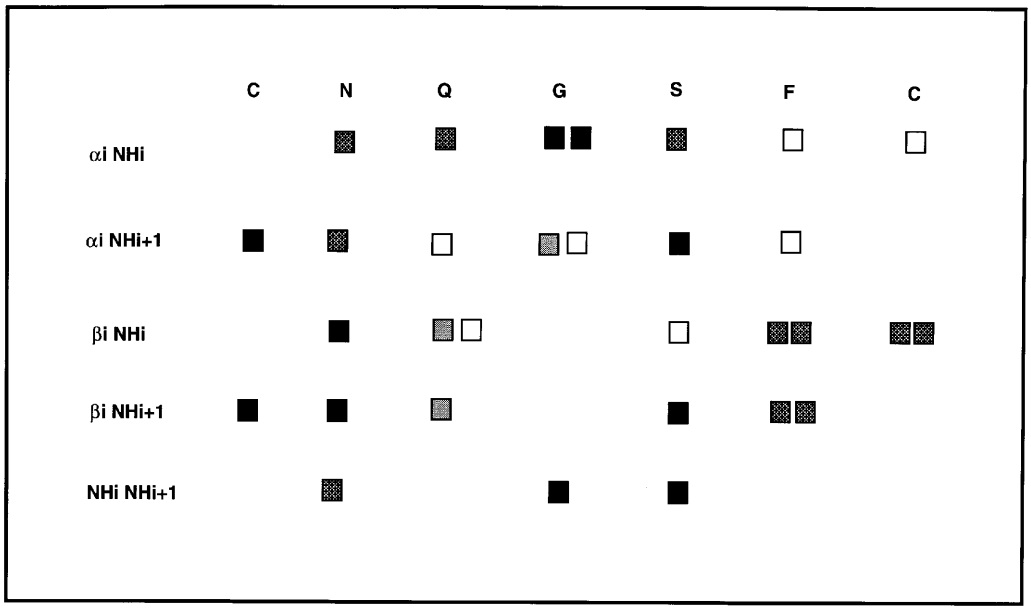

Figure 4 NOE connectivity pattern observed for CNQGSFC backbone and β protons, measured from ROESY spectra. ■ ■ ■ indicate respectively strong, medium and weak intensities. Empty squares identify cross peaks whose integrals were undetermined by spectral overlap.

interproton distances within the type II β-turn moiety. The overlap between S^5 Hb and G^4 Ha geminal hydrogens made the calculation of the exact interproton distances from the NOE intensities impossible [27]. Hence, a generic range of 2–4 Å was applied to all the proton pairs linked by Overhauser effects. A more restrictive set of molecular constraints was adopted for the NQ segment where the characteristic proton–proton distances of type II β-turn [28] were imposed.

The uniform sampling of conformational space accessible to the RI1, performed by restrained molecular dynamic and energy minimization, yielded a series of closely related molecules (Figure 5) with the RMS deviations of backbone atoms lower than 1.5 Å. Violations of NMR constraints were also within a range of 0.1–1 Å.

A structural comparison between the peptide conformers obtained from dynamic calculations and the corresponding exposed region in the human CD4 can be performed, as in Figure 6, where the backbone drawings of the CD4 C′–C″ loop and of the average minimized model of RI1 are shown. It is apparent that the designed synthetic molecule did not exhibit the expected shape. The native β-turn, indeed, is centred on the QGSF sequence, while in the synthetic peptide the same secondary structure element is shifted of one residue towards the amino terminus.

A reduced biological action of the peptide, owing to differences of the molecular structures, should not necessarily be expected, as protein surface loops do often correspond to highly flexible regions and limited rearrangements are possible, as in the present case, where after the interaction with Mabs or gp120 conformational changes of CD4 have been

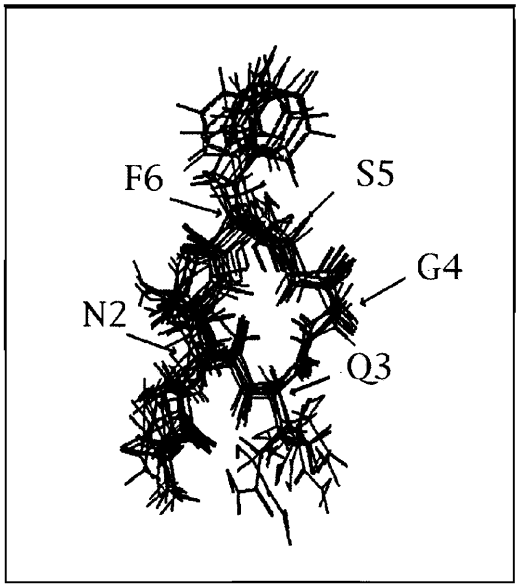

Figure 5 Low-energy conformers obtained for CNQGSFC, as described in the text.

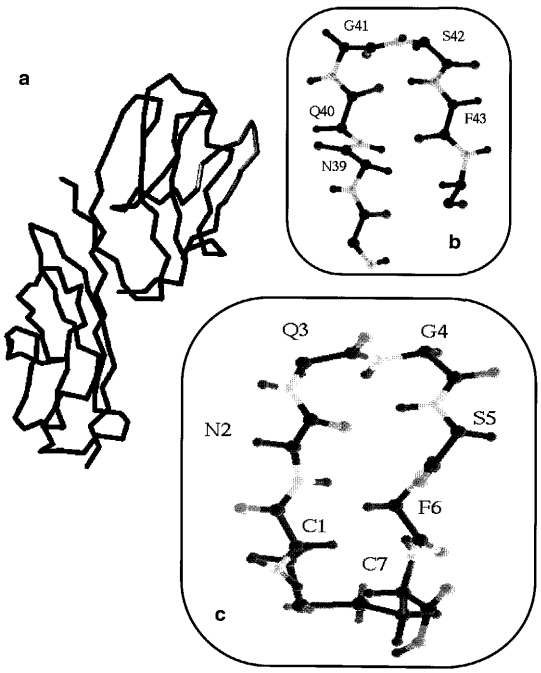

Figure 6 (a) The crystal structure of the D1 domain of CD4: the L37–K46 sequence is shown in grey. (b) A ball and stick representation of the G^{38}–L^{44} fragment. (c) the NMR-derived average conformation of the cyclic heptapeptide.

proposed [29, 30], particularly within the C′–C″ protein region [31].

A syncytia formation and lymphocyte activation assay, respectively, in the presence and absence of HIV, was carried out to asses the activities of RI1. The activity profiles indicate that this peptide induces a significant inhibition of syncytia formation in SUPT1 cells infected with HIV strain IIIB. The dose-dependent action of inhibition is reported in Figure 7. In addition, RI1 shows an evident stimulatory effect on thymidine incorporation in Jurkat cells, either in the absence or presence of a specific stimulus represented by OKT3 monoclonal antibody (data not shown).

These experimental findings strongly support the hypothesis of local structural changes in human CD4 following the virus binding. The RI1 peptide could find its ability of interfering in the gp120 interaction with a good mimicry of the functional structure of the corresponding CD4 sequence. In this respect, it is interesting to note that, from inspection of the ensemble of structures produced by restrained molecular dynamics, the F^{6} aromatic ring experiences a wide range of spatial reorientations (Figure 5).

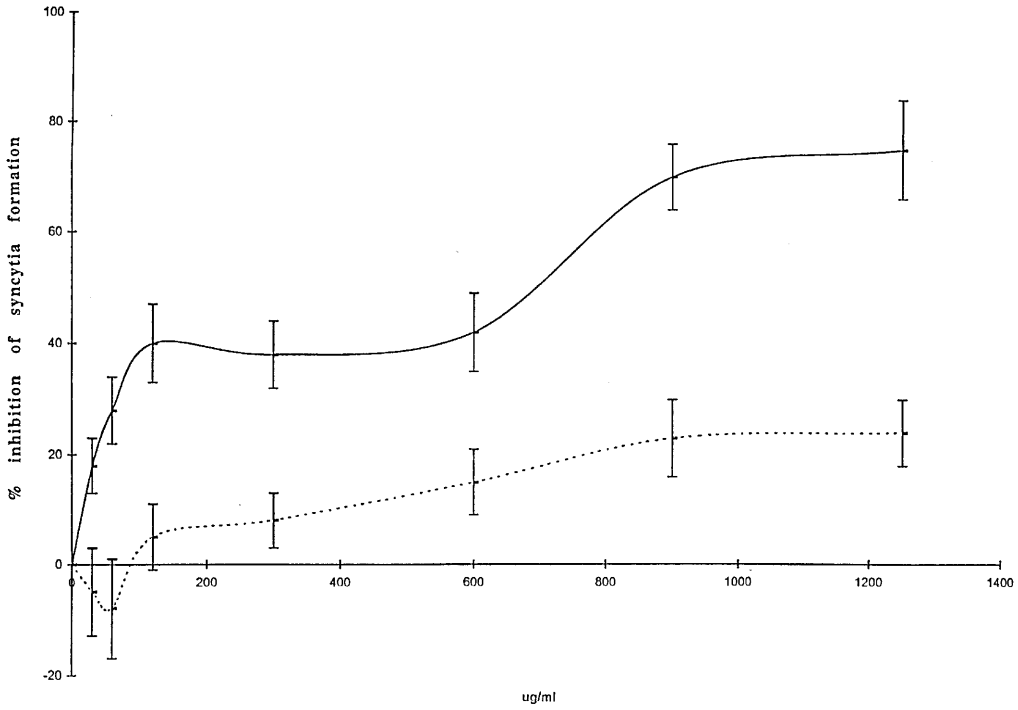

Figure 7 Percentage inhibition of syncytia formation of 10^5 Supt1 cells incubated with 10^3 HIV infected Molt4 cells (HIV strain Molt4–IIIB). Solid line indicates the dose–response effect of RI1; dashed line indicates the same effect of an unrelated control peptide.

The central role of F^{43} of human CD4 in the mechanism of the virus interaction has been outlined from the reported mutagenesis results [14, 15]. In addition, in a molecular modelling study proposed for a truncated form of gp120 [32], the insertion of CD4 F^{43} into a solvent accessible pocket of virus glycoprotein formed by gp120 W^{427}, Y^{435} and N^{230} has been suggested during the recognition process.

In this context, the high flexibility of RI1 F^{6} side chain, deduced from the absence of any sequential and long-range NOE, can favour rapid conformational transitions induced by the gp120 spatial proximity.

This conclusion emphasizes the fundamental role of internal mobility for enabling small synthetic peptides to maintain the binding properties of the cognate macromolecule. Similar conclusions have been recently proposed, dealing with the immunoreactivity of peptides as synthetic antigens: only a limited reduction of the conformational dynamics has been indicated as a suitable tool to reproduce the native epitope functionality [33].

Acknowledgements

Thanks are due to Associazione 'De Marco', MURST and CNR (Progetto Strategico Biologia Strutturale, 95.044GJ.ST74) for financial support. G. R. thanks Istituto Dermopatico dell'Immacolata, his present employer.

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