A Biochemical Approach for Detecting Interactions between Peptides from the HIV gp120 Glycoprotein and a CD4 Sequence

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Peptides selected from the HIV viral protein gp120 bind to a synthetic peptide mimicking sequence 78–89 of the human lymphocyte CD4 molecule, linked to activated Sepharose. The binding of viral fragments to the CD4 peptide-Sepharose beads was ascertained either by aid of a ninhydrin reagent or by fluorescence microscopy. A suitable alignment of these HIV peptides with the CD4 fragment showed that multiple interactions might occur between hydrophobic or charged groups of the two molecules. Although this experiment does not demonstrate that these two amino acid stretches are involved in the primary binding of gp120 to CD4 receptors, the present data suggest that the two sequences might have some kind of interaction during subsequent steps of viral infection.

Key words: Peptide-Peptide Interactions, HIV, CD4

Introduction

The CD4 molecule, a transmembrane glycoprotein found on mature T-cells, macrophages, monocytes and thymocytes (Littman, 1987), has been identified as the primary binding site for the HIV envelope glycoprotein gp120 (Clayton *et al.*, 1989; Mizukami *et al.*, 1988): this interaction appears to be the first step for the virus entry into the cell, which ultimately leads to the colonization of the lymphocyte and then to the breakdown of the immune system in AIDS.

The CD4 protein consists of four extracellular domains, a membrane-spanning region, and a cytoplasmatic tail (Leahy, 1995). Recent studies (Kwong *et al.*, 1998) have shown that the CD4 region responsible for the viral protein binding is comprised within residues 25 and 64. On the contrary, the region spanning residues 74 to 95, previously indicated as the major contact point between the two proteins (Lifson *et al*, 1988; Shapira-Nahor *et al*, 1990), do probably play only a secondary role in the binding of the viral protein, but might eventually participate in following steps of viral infection, as fusion and virus interalization.

It has been reported that short linear synthetic peptides derived from the region 410–440 of the viral protein are apparently capable of binding to CD4 receptors of intact lymphocytes, as they moderately inhibit the subsequent binding of the viral gp120 glycoprotein to the cells (Pugliese *et al.*, 1997; Chersi *et al.*, 2000b). The exact contact sequence within the CD4 receptor remains however unknown.

A fast biochemical method for detecting interactions between peptides selected from the viral gp120 proteins and the CD4 receptors might be the incubation of fluorescent peptides, selected from gp120, with CD4+ cells, and measuring their fluorescence by FACScan analysis. Unfortunately, the hydrophobic nature of reporter groups on the peptides causes massive unspecific adsorption of the fragments onto the hydrophobic lipid bilayers of cells, thus masking the specific binding (Pugliese, personal communication).

Thus, a different approach was studied: A 12-mer peptide encompassing residues 78–89 of CD4 (Table I) was first linked, through its *N*-terminal amino group, to Sepharose (Seph); then beads were allowed to react with a panel of small peptides, some of which derived from the gp120 viral protein. Some of these fragments beared a fluorescent reporter group attached to a side chain. A panel of unrelated peptides, fluorescent or not, was used as a control.

Interactions between the fragments were ascertained, for fluorescent peptides by direct examina-

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Abbreviations: ACN, acetonitrile; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline.

Table I. Sequence of the CD4 and gp120 regions presumably involved in interactions.

gp 120 (414–430)	E G S N T I T L P C R I K Q F N M
CD4 (75–96)	K N L K I E D S D T Y I C E V E D Q K E E V

PEPTIDE BRIE: (CD4 sequence 78–89)

β R I E D S D T Y I C E V

In peptide BRIE, deduced from the CD4 sequence (pos. 78–89) and used to prepare peptide-Sepharose beads, a beta-alanine (β) was added at the amino terminus, in order to introduce a spacer between the gel matrix and the fragment, and Lys1 was replaced by an arginine, in order to remove the reactivity of the ε-amino group, while keeping the positive charge.

tion of beads by aid of fluorescence microscopy, or for unmodified peptides by treatment of beads with a ninhydrin reagent and by measuring the extinctions of supernatants at 570 nm.

Materials and Methods

Peptides

Peptides (Tables II and III) were synthesized by aid of a Vega semiautomatic Peptide Synthesizer, model 1000 (Phoenix, AZ, USA) and using essentially, although not exclusively, the Fmoc technique for chain assembly (Atherton *et al.* 1978). Protected amino acids were purchased from Inalco-NovaBiochem (Milano, Italy). At the end of the syntheses, Fmoc-peptides were cleaved from the resin and deprotected by aid of TFA/ani-

Table II. Sequence of fluorescent peptides deduced from the gp120 sequence (GP9 and GP12), and of unrelated peptides used as controls (the first three are labelled with rhodamine, the second three with fluorescein).

GP9	K*N T I T L P C R I
DA	K A K*N D T H Q Q D I
TB10	K*S E E I S S E D D E L
GP12	K*E G S N T I T L P C R I
48	K*L Y Q G C Q G V
18C	F L P S D C* F P S V

(DA, TB10, 48 and 18C are control peptides)

In the sequences of fluorescent peptides, an asterisk (*) indicates the residue that bears the reporter group on the side chain. Peptides GP9 and GP12 have an additional *N*-terminal Lys. Tagging of peptides was selectively performed "in synthesis" at side chain groups of lysines (Chersi *et al.*, 1997, 2000a). Peptide 18C was labelled in synthesis at the Cys sulfhydryl group (Chersi, unpubl. results).

When Seph-BRIE beads were allowed to react with the above fluorescent peptides, only GP9 and GP12 gave highly fluorescent samples, indicating interactions between the two fragments (data not shown).

sole/mercaptoethanol, precipitated with cold ethyl ether, and recovered by centrifugation. Peptides were then purified by conventional column chromatography on Sephadex G25 superfine (in 0.02 M ammonia or 0.1% formic acid), followed by reverse-phase HPLC, with linear water/ACN gradients. Whenever necessary (Cys-containing peptides), mercaptoethanol, at a final concentration of 1 mM, was added to peptide solutions in order to prevent oxidation of cystein sulfhydryl groups or dimerization. Peptide sulfhydryl groups were detected and measured by aid of the Ellman's reagent (Ellman, 1969). The correct amino acid composition of peptides was ascertained by amino acid analysis. All peptides had a purity of 92% or more, and were kept lyophilized at − 20 °C until used.

Most fluorescent peptides (Table III) beared the fluorescent reporter group linked to ε-groups of lysine (Chersi *et al.*, 1997, 2000a): peptide 18C was labelled "in synthesis" at the SH group of Cys6 (Chersi, unpubl. results).

Peptide-Sepharose beads

Sepharose-BRIE (Seph-BRIE) peptide beads were prepared from 2 g of CNBr-activated Sepharose (Amersham-Pharmacia, Uppsala, Sweden, Prod. 17-0981-01), swollen and treated for 20 min with 0.001 M HCl on a glass filter, and 3 mg of peptide BRIE (Table I) corresponding to the stretch 78–89 of the CD4 molecule (Maddon *et al.*, 1985), with the following substitutions:

i) a beta-alanine was added at the amino terminal, in order to form a spacer between the beads and the peptide to be linked.

ii) Lys1 was replaced by an arginine, in order to eliminate the ε side chain amino group.

The reaction (in 6 ml 0.1 M sodium bicarbonate containing 0.005 ml mercaptoethanol) was allowed to proceed for 3 h at room temperature in a

rotation wheel. After low-speed centrifugation, ethanolamine (0.1 ml in bicarbonate containing 0.001% of mercaptoethanol) was then added to block residual reactive groups of the resin. The resin was kept in suspension (5 ml packed gel, 10 ml 1:1 diluted PBS containing traces of mercaptoethanol) at 4 °C. Assuming complete binding of the peptide, being the CNBr-activated Sepharose in large excess, the concentration of peptide BRIE in the conjugate suspension (15 ml) was estimated to be approx. 0.1 mg/0.5 ml.

The peptides to be tested (Tables II and III), each in the amount of 0.3 mg, were dissolved in 1:1 PBS (2 ml), and added to 0.5 ml Sepharose-BRIE suspension. The reaction was allowed to proceed in capped 5 ml-test tubes at room temperature on a rotating wheel. After 6 h, suspensions were centrifuged several times with 1:1 PBS, and kept at 4 °C.

Fluorescence assay

Beads treated with fluorescent peptides were examined directly in a fluorescence microscope. Two different methods were employed: i) by cytospin centrifugation and ii) by seeding on glass slides. Samples were then analyzed by using a Nikon Microphot fluorescence microscope (Nikon Italia, Rome, Italy) equipped with FITC and TRITC filters at the same magnification (50 ×). As results obtained were very similar, only data of the second procedure will be briefly described.

Ninhydrin assay

When dealing with non-fluorescent peptides, beads were treated with a ninhydrin reagent (5% ninhydrin in ethanol, 0.4 ml; 40% phenol in etha-

nol, 0.2 ml; 0.02 M KCN in pyridine, 0.1 ml), heated at 95 °C for 8 min, diluted with 1 ml 50% ethanol in water, and read in a colorimeter at 570 nm. The same amount of adsorbent was used as a negative control. The ninhydrin test was based on the fact that Seph-bound peptide BRIE did not possess free amino groups, as its amino terminal was linked to the matrix, while the original Lys1 of the CD4 fragment had been substituted by an arginine. Thus, no colorimetric reaction was expected. The slight colour developed by control Seph-BRIE beads by treatment with the ninhydrin reagent was used as background value.

On the other hand, any bound peptide possessed at least the free alpha amino group, thus, the color intensity (extinction) measured after treatment with the ninhydrin reagent was assumed to be proportional to the amount of bound peptide.

Results and Discussion

In Table III, the ninhydrin assay performed on Seph-BRIE beads incubated with non-fluorescent peptides is reported. There is a clear indication that both two gp120-derived peptides and some of the structurally-related peptides bind to the CD4 fragment linked to the beads, while control peptides do not. Seph-BRIE beads remain colorless, as they do not possess amino groups available for reaction.

Also fluorescence microscopy of 4 peptide-treated Seph-BRIE beads reveals marked differences between different samples: in fact, while a low fluorescence emission was detected for control peptide 48, markedly positive beads were found for peptide GP12 (green blobs), and for peptide

Table III. Sequence of gp120 peptides and related fragments F, 96, QM9, of control peptides 92K and A3 and extinctions at 570 nm as obtained by the ninhydrin test on Seph-BRIE-peptide complexes.

Name	Sequence	Extinctions at 570 nm
GP9	N T I T L P C R I	0.330 (0.310–0.350)
GP12	E G S N T I T L P C R I	0.380 (0.360–0.400)
F	N T I T L P S R I	0.200 (0.180–0.220)
96	E F G T S S C R L	0.360 (0.335–0.385)
QM9	E Q L I M Q C R I	0.320 (0.305–0.335)
92K	D I L P P K L F V	0.120 (0.105–0.135)
A3	G E T G D C D S K V G	0.140 (0.130–0.150)
Seph-BRIE beads		0.110 (0.100–0.120)

The ninhydrin assay was performed in duplicates as reported in the text. 92K and A3 are control peptides.

GP9 (red blobs). A very low red fluorescence was detected in the other control sample (peptide DA) (data not shown).

Peptide-protein interactions are common: one of the most striking examples is the binding of nonapeptides by class I histocompatibility antigens (Di Modugno *et al.*, 1996): Although only two (occasionally three) residues function as main anchor residues, the particular folding of the protein shapes a well-defined groove where the peptide fits perfectly, and this results ultimately in an increased stability of the HLA-peptide complex.

Peptide-peptide interactions are less common and quite difficult to detect: In general, few interactions between hydrophobic residues or charged groups within two small fragments do not provide sufficient strength to form a complex sufficiently stable to be isolated in the absence of a three-dimensional structure of one of the two molecules. However, in the case of peptides BRIE and GP9/GP12, we faced an extremely favourable situation. By suitably aligning sequences of the peptide from CD4 and that of the fragments from the viral gp120 protein, in the region 414–425,

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\begin{array}{l}
\quad 1 \qquad\qquad\qquad 12 \\
\mathbf{O}-\beta\text{RIEDSDTYICEV} \\
\quad\ \text{EGSNTITLPCRI}
\end{array}
$$

we presume that several kinds of interactions might occur as the two peptides come into close contact: Arg1 and Glu11 of the first peptide face exactly Glu1 and Arg11 of the second peptide, giving strong charge interactions. In addition, hydrophobic interactions would also occur between Tyr8 and Val12, with Leu8 and Ile12 of the viral peptides.

It should be admitted that this extremely favourable situation, where so many hydrophobic residues or charged groups in the two fragments can interact, should be considered as quite rare. In addition, and probably very important, by this alignment the two Cys residues would face exactly each other, permitting thus, by slight oxidative processes, the formation of a stable disulfide bridge. Peptide F, with a serine replacing Cys at position 3, gives a lower binding (Table III). In addition, when peptide BRIE is linked to the beads in the form of the dimer, a very remarkable reduction (70%) of the binding of peptide GP9 is observed (data not shown).

Although it is difficult to estimate the effective contribution of the domain 74–95 of the CD4 molecule in the binding of the viral protein, as compared to the main active sequence 25–64, the present biochemical data seem to indicate that *in vitro* and possibly at a certain step during the *in vivo* HIV infection, this amino acid stretch on the CD4 molecule might bind this well-defined sequence within the gp120 molecule and confirm data of a previous publication obtained by using HIV gp120, synthetic peptides, and CD4+ cells (Chersi *et al.*, 2000b). The 12-mer fragment GP12 seems to be the best binder for the CD4 BRIE bead, followed by P96 and GP9: Indeed, this former fragment has an additional glutamic acid residue which can then interact with the arginine of the bead. As both peptides may be assumed to be random-coiled, there is no sterical hindrance to possible interaction, being the only rigid structure, the Sepharose bead, kept at a certain distance from the peptide by the beta-alanine bridge. Working with intact cells, however, the three-dimensional conformation of the CD4 molecule might prevent long peptides from adapting themselves to a particular rigid folded structure.

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