Use of a constrain phage displayed-peptide library for the isolation of peptides binding to HIV-1 nucleocapsid protein (NCp7)

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Abstract It has been shown that peptide libraries are powerful tools for the identification of peptides showing new binding specificity. This technology was applied to the isolation of peptides binding to HIV-1 nucleocapsid protein (NCp7). Three different prolin reach peptide sequences, interacting with NCp7, were isolated, from a constrained phage displayed-peptide library of 10^8 independent clones. The three peptide sequences, isolated from the peptide library, were shown to bind NCp7 in the region 30–52. Moreover, two of them share the PP-(D/E)R consensus sequence.

Key words: Peptide library; Phage; Biopanning; HIV-1; Nucleocapsid protein; Synthetic peptide

1. Introduction

Peptide display technology permits the construction of vast collections (libraries) of peptides of random amino acid sequences [1,2]. Phage displayed-peptide libraries appear to be powerful tools to isolate peptide sequences binding to target molecules. It has been shown that phage displayed-peptide libraries can be used successfully in the identification of epitopes and mimotopes recognized by antibodies [3–6]. Furthermore, the phage displaying technology has been used for the isolation of protein antagonists and inhibitors [7–9]. In this paper, HIV-1 nucleocapsid protein (NCp7) was used as target for the screening of a constrained phage displayed-peptide library [5]. HIV-1 nucleocapsid protein has two sequential repetitions of 14 amino acids containing cysteines and histidines arranged as C-X2-C-X4-H-X4-C [10,11], and mediates the viral RNA dimerization process [12]. HIV-1 nucleocapsid protein has been detected in HIV-1 virions in two forms, NCp15 and NCp7 [13] and the generation of NCp7 is necessary for specific RNA packaging [14]. We focus our attention on the isolation of NCp7 ligands because of their potential interest for the development of new compounds that, interfering with NCp7 functions, might reduce the infectivity of neo-synthesized virions. Here, we describe the collection of peptide ligands we have isolated biopanning a constrained phage displayed-peptide library of 10^8 independent clones against NCp7 of HIV-1.

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Abbreviations: HIV-1, human immunodeficiency virus; BSA, bovine serum albumin; pVIII, M13 capsid protein pVIII; $C(X)_9C$, nine random amino acids surrounded by two cysteins; Ap, ampicillin; TU, transforming units; Ap^R, ampicillin resistance; NCp7, HIV-1 nucleocapsid protein p7; PBS, NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 10.14 mM, KH_2PO_4 1.76 mM; RT, room temperature; OD, optical density; P/N, ratio between $OD_{590\ nm}$, associated to NCp7 coated wells and $OD_{590\ nm}$, associated to BSA coated wells.

2. Materials and methods

All chemicals were purchased from Sigma (USA). All the chemically synthesized peptides were kindly supplied by Dr. B. Roque and Dr. H. de Rocquigny [15]. The constrained phage displayed-peptides library was kindly gifted by Dr. F. Felici [5].

2.1. Biopanning of a pVIII-$C(X)_9C$ phage displaying library against a chemically synthesized HIV-1 nucleocapsid protein (NCp7)

Peptides binding to HIV-1 nucleocapsid protein, NCp7, were selected from of a constrained phage displayed-9-mer peptide library [5] performing four biopanning steps against a chemically synthesized NCp7 [15], adsorbed on a plastic surface. NCp7 (2 ml), was adsorbed in a 25 cm^2 cell culture flask (Costar, USA; 18 h at 4°C), at a concentration of 5 μg/ml (flask A) or 1 μg/ml (flask B) in 50 mM carbonate buffer pH 10.0. Subsequently, flask A and B were overcoated (1 h, 37°C) with a solution containing PBS/1% no fat dry milk (Carnation, USA; 3 ml). BSA was coated , at a concentration of 5 μg/ml (flask C) in 50 mM carbonate buffer pH 10.0, into a 25 cm^2 cell culture flask as described for NCp7. The overcoating procedure was also done for flask C. Flasks A, B and C were washed four times with PBS (4 ml, 10 min, RT) and stored desiccated at −20°C, until needed. The phage displayed-peptide library (2 ml) was preadsorbed onto flask C (1h, 37 °C). The preadsorbed library was then transferred in flask A (biopanning 1 and 2) or flask B (biopanning 3 and 4) (4 h, RT). Furthermore, the unbound phages were removed, initially with 10 washes of PBS/0.05% Tween-20 (4 ml, 10 min) and then with 4 washes of 0.1 N HCl/glycine pH 2.2 (2 ml, 5 min). Phages, still bound to the plastic matrix coated with NCp7, were rescued by addition, to the flask, of 0.5 $OD_{590\ nm}$ TG1 cells (Pharmacia, S; 1 h, 37°C). The cells were, then, plated on LB agar supplemented with 50 μg/ml ampicillin and left at 37°C (18 h). Colonies (bacterial sublibrary) were scraped from the plate and suspended in LB medium (4 ml). The bacterial sublibrary were superinfected as described by F. Felici [5], to produce the phage sublibrary to be used in the next biopanning.

2.2. Single phage screening procedure

10^9 Ap^RTUs (100 μl) of each NCp7 binding phage, derived from the 3rd and the 4th biopanning, were diluted in PBS/1% no fat dry milk (100 μl). Two aliquots of the phages were incubated (1 h, 37°C), in parallel, into microtiter wells (Costar, USA) respectively coated with NCp7 and BSA. The unbound phages were removed, initially, with 10 washes of PBS/0.05% Tween-20 and then with 1 wash with 50 mM citrate buffer, pH 4.0 (30 min, 25°C). Phages, still bound to the wells, were rescued by addition of TG1 cells (100 μl, 1 h, 37°C), to each well. 20 μl of each bacterial suspension were transferred to new microtiter wells containing of LB medium (200 μl), supplemented with 100 μg/ml ampicillin. The cultures were grown under vigorous shacking (18 h, 30°C, 250 rpm/min). The culture turbidity was measured at 590 nm. All phages showing a P/N value higher than 2 were considered NCp7 binding phages.

2.3. NCp7 binding titration assay

NCp7 binding phages were diluted in PBS/1% no fat dry milk to obtain the following Ap^RTUs in each well: 5×10^{10}, 1×10^{10}, 5×10^9, 1×10^9, 5×10^8, 1×10^8, 5×10^7, 1×10^7, 5×10^6, 1×10^6. Each phage concentration (100 μl) was incubated (1 h, 37°C) into two microtiter

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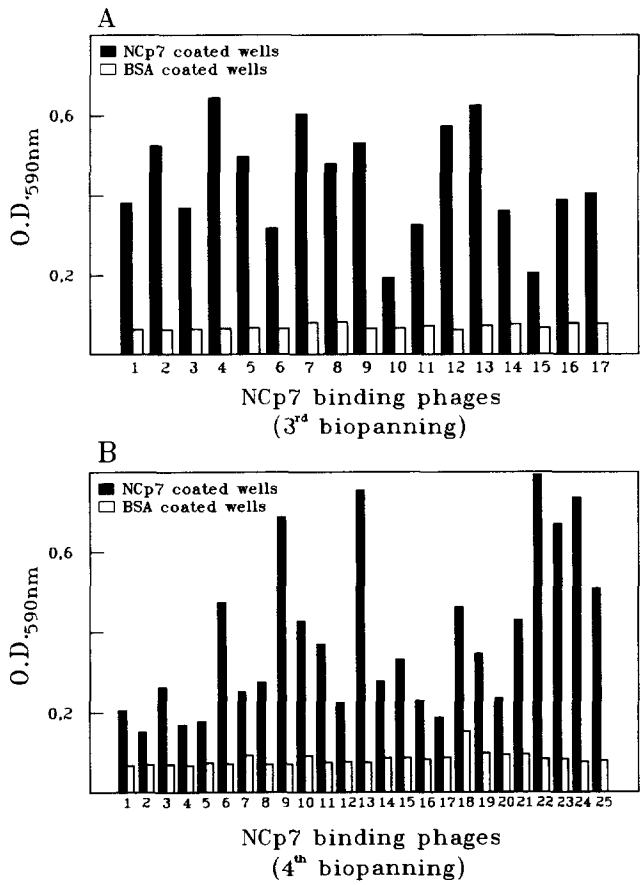

Fig. 1. Single phage screening procedure. (A) NCp7 binding phages isolated from the analysis of 48 phages from the 3rd biopanning. (B) NCp7 binding phages isolated from the analysis of 96 phages from the 4th biopanning.

wells (Nunc, USA) coated respectively with NCp7 and BSA. Unbound phages were washed out as described in the single phage screening procedure. Phages, still bound to the wells were rescued by addition of TG1 cells (100 μl, 1 h, 37°C), to each well. Subsequently, 50 μl of each bacterial culture were plated on LB agar plates, supplemented with 100 μg/ml ampicillin (18 h, 30°C). Colonies (Ap^RTUs), obtained from each phage concentration and corresponding to NCp7 or BSA coated wells, were counted. The number of colonies associated to BSA coated wells were subtracted, for each dilution point, from the number of colonies associated to NCp7 coated wells. The experiments were repeated three times and. every time, we observed the same binding behavior.

2.4. Interaction of NCp7 binding phages with NCp7 deletion peptides

The peptides representing respectively NCp7 regions 12–53, 29–72, 21–35, 31–45 and 41–56 were coated on microtiter plate strips (8 wells, Nunc, USA), using a coating concentration of 5 μg/ml. NCp7, the deletion peptide to be evaluated (12–53 or 29–72 or 21–35 or 31–45 or 41–56), an unspecific peptide (69–89 HIV-1 p6) and BSA were coated in double, on each strip. Subsequently, the coating performance was checked using microBCA assay (Pierce, USA). Each strip was incubated with 1×10^{10} Ap^RTUs of phages pepB, C or 1×10^9 Ap^RTUs of phage pepD (1 h, 37°C). The strips were washed as described previously in the single phage screening procedure. The phages, still bound to the wells, were rescued adding TG1 cells (100 μl), in early log phase. 50 μl of each bacterial culture was plated on a LB agar plate, supplemented with 100 μg/ml ampicillin. The number of Ap^RTUs, associated to wells coated with NCp7 deletion peptides, was counted and compared with the number of Ap^RTUs, associated to NCp7 coated wells. The binding was considered positive if the amount of Ap^RTU associated to each deletion peptide was in the same order of magnitude of the Ap^RTUs associated to NCp7.

Table 1
Biopanning steps performed against NCp7 adsorbed on a plastic matrix, using a pVIII-C(X)$_9$C library (1st biopanning) and the derived sublibraries (2nd–4th biopannings)

Biopanning	Starting titer (Ap^RTUs)	NCp7 bound phages (Ap^RTUs)	NCp7 coating concentration (μg/ml)
1st	3.3×10^{10}	1.5×10^3	5
2nd	2.6×10^9	2.0×10^4	5
3rd	8.0×10^9	513	1
4th	5.0×10^9	380	1

3. Results and discussion

A constrained phage displayed-peptide library [5], built inserting at the NH_2-terminus of M13 pVIII protein 9 random amino acids surrounded by two cysteins, was used for the selection of NCp7 binding peptides. Four biopanning steps were done to isolate peptides having high affinity for the NCp7 (Table 1). The first and the second biopanning were done on NCp7, coated on a plastic matrix at a concentration of 5 μg/ml. The third and the fourth biopanning were performed on NCp7, coated on a plastic matrix at a concentration of 1 μg/ml (material and methods). The decreased coating concentration of NCp7, during the biopanning steps was used to select the phages binding more tightly to NCp7. However, it was impossible to isolate NCp7 binding phages, performing the 3rd biopanning with a NCp7 coating concentration lower than 1 μg/ml. Moreover, we rescued the phages still bound to NCp7 after the treatment with HCl-glycine buffer (pH 2.2), which is used in the standard protocols to elute the specifically bound phages from the matrix [5]. This approach has the advantage to rescue the phages tightly bound to the target molecule but it might increase the number of co-selected false positives. One by one analysis of phages from the 3rd and the 4th biopanning, showed that respectively 35% (17/48) and 25% (25/96) of the phages

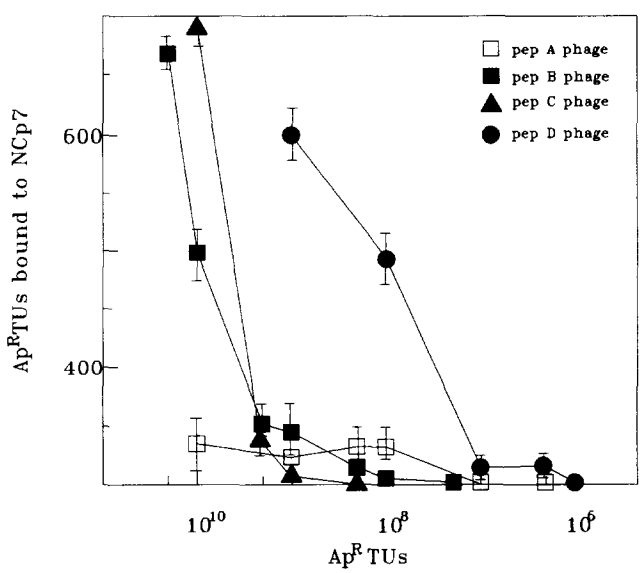

Fig. 2. NCp7 binding titration assay. The NCp7 binding behavior of phages, expressing the four peptide sequences isolated from the phage peptide library, was evaluated in dilution.

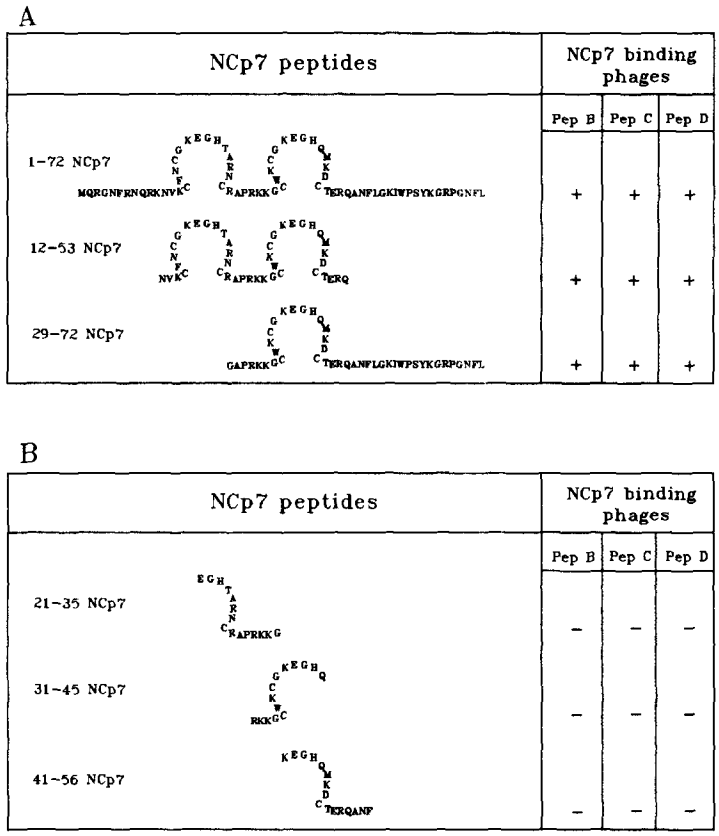

Fig. 3. (A) Phage binding on NCp7 and NCp7 deletion peptides. (B) Phages binding to partially overlapped peptides covering the region 21–56 of NCp7. In the figure the two zinc ions associated to NCp7 are not indicated because, in the coating condition used, the zinc ions are released.

were specifically interacting with NCp7 (Fig. 1A,B). This result agrees with the leak of the increment of specifically NCp7 binding phages among the 3rd and the 4th biopanning and with the growth of unspecific ligands, 6% in the 3rd and 30% in the 4th, observed between those biopannings (data not shown).

Table 2
(A) NCp7 binding peptide sequences associated to phages isolated from the 3rd and the 4th biopanning; (B) sequence homology observed between pepB and pepC sequences with the early protein EP0 from Pseudorabies virus [17]

A

Clones	Sequence of the insert	Isolates (3rd biopanning)	Isolates (4th biopanning)
pep A	C L P L V D V F G D C G	6/17	18/25
pep B	C P P E R L S L P I C G	7/17	–
pep C	C G C P P V D R C E C G	3/17	7/25
pep D	C S D V R P I P V A C G	1/17	–

B

Clones	Sequence homology			swissprot name
pep B		P P E R L S L P I		
		P P + R L S L P +		
	33	P P Q R L S L P L	41	ICP0_PRVIF
pep C		G C P P V D R C E		
		P P + R		
	33	P P – Q R	36	ICP0_PRVIF

The one by one analysis was done using a single phage screening procedure based on the detection of Amp^R *E. coli* growth associated to the presence of phages bound to the NCp7, immobilized on a plastic matrix (materials and methods). The development of this new assay was necessary since the 'ELISA phage detection system' produced by Pharmacia (S) was not sensitive enough. The DNA sequence of the 42 NCp7 binding phages showed four different peptide sequences associated to the NCp7 binding specificity (Table 2, A). Moreover, it was observed that only pepA and pepC sequences were represented in the phages isolated from the 4th biopanning (Table 2, A). The range of phage concentrations where the four sequences bind specifically NCp7 was mesured using a dilution assay (section 2). It was observed that pepB, (closed square), pepC (closed triangle) and pepD (closed circle) phages showed a dose dependent binding to NCp7 (Fig. 2). Instead, pep A phage (open square) showed a poor NCp7 binding, independent from the amount of phages used and it was considered a false positive. By comparing pepB, pepC and pepD sequences with the Swissprot data bank, using the program BLASTP [16], we observed that pep B and pep C sequences show homology with EP0 protein (ICP0_PRVIF; Table 2, B), which is a zinc finger protein, early expressed during the life cycle of the Pseudorabies virus [17]. The homology is located in a region immediately upstream to the EP0 cystein-rich zinc finger domain. Aligning pepB sequence with pepC sequence it was observed the presence of a PP-(D/E)R consensus sequence. Moreover, it was observed that pepB, pepC and pepD bind to NCp7 regions

12–53 and 29–72, suggesting that the NCp7-phage interaction involves amino acids located in the region 30–52, encompassing the APRKK linker domain and the second zinc finger (Fig. 3A). It was impossible to define more precisely the phage binding sites on NCp7 because it was not obseved any binding when shorter and partially overlapped peptides, rappresenting the region 21–56 of NCp7 were used (Fig. 3B).

In conclusion, we have isolated three peptide sequences interacting with NCp7. The phage-peptides/NCp7 interactions involve amino acids located in the 30–52 region of NCp7. Furthermore, these NCp7 binding peptides could be a potential starting point for the design of compounds interfering with the NCp7, which is very important in the process of RNA selection during the HIV-1 virion assembly [14]. Moreover, these results are an other indication that phage peptide libraries can be used not only for epitope and mimotope identification but they are an alternative way to identify peptides showing new binding specificity.

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