# Identification by Phage Display Selection of a Short Peptide Able To Inhibit Only the Strand Transfer Reaction Catalyzed by Human Immunodeficiency Virus Type 1 Integrase†

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ABSTRACT: Human immunodeficiency virus type 1 integrase catalyzes the integration of proviral DNA into the infected cell genome, so it is an important potential target for antiviral drug design. In an attempt to search for peptides that specifically interact with integrase (IN) and inhibit its function, we used an in vitro selection procedure, the phage display technique. A phage display library of random heptapeptides was used to screen for potential peptide ligands of HIV-1 IN. Several phage clones were identified that specifically bound IN. Two of the selected peptides (FHNHGKQ and HLEHLLF) exhibited a high affinity for IN and were chemically synthesized. High affinity was confirmed by a displacement assay which showed that these two synthetic peptides were able to compete with the phages expressing the corresponding peptide. These agents were assayed on the in vitro IN activities. While none of them inhibited the 3′-processing reaction, the FHNHGKQ peptide was found to be an inhibitor of the strand transfer reaction. Despite its high affinity for IN, the HLEHLLF peptide selected and assayed under the same conditions was unable to inhibit this reaction. We showed that the FHNHGKQ peptide inhibits specifically the strand transfer activity by competing with the target DNA for binding to IN. These IN-binding agents could be used as a base for developing new anti-integrase compounds as well as for structural studies of the still unknown three-dimensional structure of the entire integrase molecule.

Integration of HIV-1 viral DNA into the cell genome is a prerequisite for retroviral replication and chronic infection. Integrase (IN)[1] is the crucial virus-encoded enzyme that catalyzes the insertion of retrovirus genome into host cell chromosomal DNA (*1*, *2*). This key reaction of the retroviral cycle proceeds in different steps: (i) 3′-end processing in which IN removes two nucleotides (GT) from the 3′-end of each strand of linear viral DNA, leaving the conserved CA dinucleotide at the viral 3′-end; (ii) strand transfer during which IN covalently joins the recessed 3′-OH ends of the viral DNA to the target DNA by a transesterification reaction; (iii) 5′-end joining, a process that consists of removing the two unpaired nucleotides at the 5′-ends of the viral DNA and filling in the gaps between the viral and target DNA sequences. The partners involved in this last repair step remain to be identified.

HIV-1 IN displays three structural and functional domains (reviewed in ref *3*). The amino-terminal domain (residues 1−50) contains the conserved HHCC motif that binds one atom of zinc (*4*). This region is involved in protein−protein interaction and contributes to the specific recognition of viral DNA ends (*5*). The core or catalytic domain (residues 50−212) contains the highly conserved D,D(35)E motif present in all retroviral integrases. The carboxy-terminal domain (residues 213−288) is the least conserved IN domain. It is involved in DNA binding and IN oligomerization, which seems necessary for the integration process (*6*, *7*).

As HIV-1 IN is essential for productive retroviral infection, it constitutes a potential target for novel antiviral chemotherapy. In contrast to HIV-1 reverse transcriptase and protease, extensively used as targets in the combined therapy strategy, few inhibitors of IN described until now seem to behave as potential therapeutic agents. This lack is partly due to the difficulties encountered in structural studies because of the low solubility of the whole enzyme, and to insufficient information concerning the biochemical mechanism of proviral integration. Various classes of HIV-1 IN activity inhibitors such as diketo acids (*8*−*11*), styrylquinolines (*12*), guanosine quartet oligonucleotides (*13*), and chicoric acid derivatives (*14*, *15*) have been identified in vitro and exhibited inhibitor effects on HIV-1 replication cycles in cells (reviewed in refs *16*−*18*). Among these promising inhibitors of HIV-1 IN, diketo acids represent a novel and selective class of compounds as their effect on integration resides in their specificity for the strand transfer step (*8*, *9*).

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[1] Abbreviations: IN, integrase; LTR, long terminal repeat; ODN, oligodeoxynucleotide.

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Moreover, mutated integrases were selected for their resistance to diketo acid derivatives, proving that the integration step was targeted in infected cell cultures (*19*−*21*).

Rapid development of chemical methods for peptide synthesis opened the way for the use of these molecules as antiviral agents on infected cells. While therapeutical drugs were previously identified by trial and error, low molecular weight inhibitory peptides are currently being isolated by large-scale screening of peptide libraries. Several strategies have been developed: (i) synthetic peptide combinatory libraries; (ii) a two-hybrid system; (iii) phage display (reviewed in ref *22*).

By using a synthetic peptide combinatorial library, Puras Lutzke et al. have reported the identification of a hexapeptide (*23*) that inhibits both IN-mediated in vitro reactions with $IC_{50}$ values in the micromolar range.

The two-hybrid system allowed us to isolate a 33-mer peptide ($I_{33}$) which bound tightly to HIV-1 IN. The peptide $I_{33}$ inhibited both the in vitro 3′-end processing and strand transfer activities. Further analysis led to selection of a shorter peptide, EBR28, that presents a significant antiviral effect when assayed on HIV-1-infected human cells (*22*, *24*, *25*).

The phage display technology has proven to be a valuable tool to probe protein−ligand interactions (*26*, *27*). Phage display is an in vitro selection procedure in which the coding region of a peptide or a protein is generally fused to a bacteriophage coat protein, resulting in display of several copies of the fused protein on the surface of the phage, while the DNA encoding the fusion domain is confined within the virion. The main advantage of this selection over other technologies is the ease of screening large numbers of random short peptidic sequences, according to their affinity for a target protein. Thus, this approach may help to identify inhibitory peptide leads, thereby allowing an accelerated process for designing anti-HIV small molecules.

To identify and characterize novel short peptides able to interact specifically with HIV-1 IN, we used a phage display heptapeptide library. A crucial advantage of phage display technology is the direct link between the experimental phenotype and its encapsidated genotype, which allows the evolution of the selected binders into optimized molecules. In this study we identified novel high-affinity peptides for HIV-1 IN. Among several peptides that bind to IN, we focused our interest and characterized two peptides. Interestingly, one of the peptides, FHNHGKQ, exhibited a specific inhibitory effect only on the strand transfer IN activity. Binding experiments between IN and its substrates strongly suggest that this peptide inhibits strand transfer reaction by competing with the target DNA, probably by preventing its binding to a specific site on the enzyme. These results show that peptides generated by phage display could provide promising compounds to design new agents against HIV-1 IN and to clear mechanisms involved in the reaction catalyzed by this enzyme.

## MATERIALS AND METHODS

*A. Materials*. Phage display peptide library Ph.D.-7 was purchased from New England Biolabs (Beverly, MA). It consists of randomized linear heptamer peptides, fused to the coat protein pIII of M13 via a flexible linker, GGGS, and expressed on the phage surface in five identical copies. Peptides were purchased from MWG-Biotech AG. They were synthesized with the linker sequence GGGS and an amide group at the C-terminus. Peptides were dissolved in dimethyl sulfoxide (DMSO), and diluted in water−DMSO when needed.

*B. Methods. 1. Purification of Recombinant HIV-1 Integrase. i. Untagged IN Expressed in Yeast.* Purification was performed essentially as previously described (*28*, *29*). After purification, fractions containing IN activity were pooled and concentrated by ultrafiltration (Centricon Millipore), followed by addition of 7 mM Chaps. Purified integrase was kept at −80 °C.

*ii. His-Tagged IN*. pET-15b(His-$IN_{1-288}$), pET-15b-(His-$IN_{50-212}$), and pET-15b(His-$IN_{50-288}$) expression vectors encoding His-tagged truncated INs (a generous gift of J. F. Mouscadet, UMR-8532 CNRS, ENS Cachan, Villejuif, France) were used to express and purify His-tagged INs in *Escherichia coli* BL21(DE3) as described by Leh et al. (*30*) with minor modifications: all buffers contained 7 mM Chaps, and $ZnSO_4$ was omitted during elution. Expression was induced by IPTG (1 mM) at an initial OD of 0.8. Cultures were incubated for 3 h at 37 °C, after which the cells were centrifuged. The cell pellet was resuspended in ice cold buffer A (20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 2 mM DTT, 7 mM Chaps, 10 mM imidazole, 100 mM PMSF, and protease inhibitor cocktail tablets (Roche)), treated with lysozyme for 1 h on ice, and sonicated. After centrifugation (30 min at 10000 rpm) the supernatant was filtered on membranes (0.45 $\mu$m) and loaded onto a HiTrap chelating column charged with nickel ions (1 mL, Amersham Pharmacia Biotech). The column was washed with buffer A and 100 mM imidazole and eluted in a single step with buffer A and 500 mM imidazole on a liquid chomatography system (Äkta Prime). After elution, the His-tagged IN fusion proteins ($\Delta$C and $\Delta$N) were cleaved using biotinylated thrombin (Novagen) and dialyzed overnight against 20 mM Tris−HCl, pH 8.0, 500 mM NaCl, 2 mM DTT, and 10% glycerol. Thrombin was then captured by incubation with streptavidin magnesphere paramagnetic particles (Promega). Chaps was added to a final concentration of 7 mM to fractions containing IN.

*2. Panning a Peptide Library Displayed on a Filamentous Phage*. The random peptide phage display library Ph.D.-7 was applied to purified IN previously coated overnight in Nunc Immunosorp 96-well plates at a concentration of 100 $\mu$g/mL in 100 $\mu$L of 0.1 M Hepes, pH 7.6. The wells were blocked for 1 h with 200 $\mu$L of BSA (1%) in the same buffer and rinsed with wash buffer (PBS, 0.1% Tween 20) before addition of the phages. A total of $2 \times 10^{11}$ phages of the original library were diluted in 100 $\mu$L of PBS−Tween (0.1%), applied to a single well, and allowed to bind to IN or BSA (1%) at room temperature for 1 h. The plates were washed extensively (10 times) to remove unspecifically bound phages. Bound phages were eluted by treatment with 100 $\mu$L of 0.2 M glycine−HCl, pH 2.2, 1 mg/mL BSA and gentle shaking for 10 min. The eluate was neutralized at pH 7 with 1 M Tris−HCl, pH 9.1 (150 $\mu$L/mL of eluate); 1 $\mu$L was used for titration on log-phase *E. coli* ER 2738 cells. The remainder was amplified in the same *E. coli* strain according to the protocol provided by the manufacturer. Then $2 \times 10^{11}$ phages were reapplied to IN or BSA in new wells

for the next more stringent round of selection by increasing the percentage of Tween 20 in the wash buffer (from 0.1% to 0.5%). After 3 rounds of selection and amplification, 20 individual clones of each selection were automatically sequenced on an ABI Prism 310 genetic analyzer using the −96 gIII primer (5′-CCCTCATAGTTAGCGTAACG-3′) and a Big Dye terminator cycle sequencing kit (PE Biosystems).

*3. Phage Binding Assays*. Binding studies were performed using enzyme-linked immunosorbent assay (ELISA) in the same conditions as the selection with IN. Ten-fold serial dilutions of phages (from $10^{13}$ to $10^{8}$ phages/mL) were applied to the wells coated with IN (100 $\mu$g/mL) or deleted IN (20 $\mu$g/mL) and incubated for 2 h at room temperature. Unbound phages were removed by washing the plates six times with PBS−Tween (0.5%). An anti-M13 antibody conjugated with horseradish peroxidase (HRP) was added to detect phage interaction with INs. Peroxidase was monitored with an Amplex Red hydrogen peroxide assay kit (Molecular Probes BioProduct) by emission of fluorescence using a fluorescence multiwell plate reader, Cytofluor II (PerSeptive Biosystems).

*4. Competitive ELISAs*. Coated IN was incubated with a solution containing a constant phage concentration which corresponded to 50% fixation to IN in the binding assays, and increasing concentrations of the synthetic peptide selected as described above. Phage binding was monitored by anti-M13 antibody.

*5. Integrase Activity Assays*. Substrates used to assay the 3′-end processing and strand transfer reactions were oligonucleotides (ODNs) of different sizes containing sequences derived from the U5 end of HIV-1 LTR (*31*). The 3′-processing assay was done with 10 nM 5′-$P^{32}$-labeled ODN-1 (5′-GTGTGGAAAATCTCTAGCAGT-3′) annealed to its complementary strand ODN-3 (5′-ACTGCTAGAGATTTTC-CACAC-3′). The strand transfer reaction was performed using either 10 nM 5′-$P^{32}$-labeled ODN-2 (5′- GTGTG-GAAAATCTCTAGCA-3′) annealed to ODN-3 or a 10 nM concentration of the double-stranded ODN used for the 3′-processing reaction. In all assays the standard reaction mixture contained 20 mM Hepes, pH 7.5, 10 mM dithiothreitol, 7.5 mM $MnCl_2$, 0.05% Nonidet-P40, and 300 nM IN in a final volume of 20 $\mu$L. The corresponding 5′-labeled ODNs were added to the reaction mixture and incubated at 37 °C for different times depending on the assay. Reactions were stopped by adding a volume of loading buffer (95% formamide, 20 mM EDTA, 0.025% SDS, 0.025% bromophenol blue, and 0.025% xylene cyanol) to obtain a final concentration of 100000 cpm/well. After heating at 90 °C for 5 min, the reaction products were subjected to electrophoresis on 12% polyacrylamide gels with 7 M urea in Tris−borate−EDTA (TBE), pH 7.6, and autoradiographed. The gels were analyzed using an NIH-image device.

*6. Inhibition Experiments*. Peptides were dissolved in DMSO, and successive dilutions were made in $H_2O$−DMSO. Different peptide concentrations were preincubated with 300 nM IN for 20 min at 37 °C in the standard reaction mixture but containing a final concentration of 1% DMSO. Reactions were started by adding the DNA substrate as described above for the IN assays. The concentration producing 50% inhibition ($IC_{50}$ value) was determined by plotting the inhibitor concentration versus the percentage of IN residual activity.

Table 1: Amino Acid Sequences of Phage-Displayed Peptides Selected against IN or BSA[a]

| protein target | phagotope sequence | no. of individual phages isolated | protein target | phagotope sequence | no. of individual phages isolated |
|---|---|---|---|---|---|
| IN | **FHNHG**AA | 3 | BSA | | |
| | **FHNHG**KQ | 2 | | NPRLYE | 4 |
| | **FHNHG**IL | 1 | | TTYSRFP | 3 |
| | **FHNHG**AT | 1 | | AEPVAML | 2 |
| | **FHNHG**ST | 1 | | VPTGYKP | 2 |
| | **FHNHG**AP | 1 | | ASSRTPS | 1 |
| | **FH**G**HG**LY | 1 | | HFWNRPL | 1 |
| | **HIEHLI**A | 1 | | FHQNWPS | 1 |
| | **HLEHLL**F | 1 | | HWGMWSY | 1 |
| | PFF**HLI**G | 1 | | HAWNYIF | 1 |
| | RLFTWE | 2 | | NSHAIYP | 1 |
| | SSLPLRK | 2 | | SLLSSPQ | 1 |
| | STPIPAP | 1 | | SPYHTQP | 1 |
| | STFTHPR | 1 | | LPPNPTN | 1 |

[a] The total number of phagotopes sequenced was 20 for each selection. Phagotopes are listed according to their relative abundance. The consensus sequence **FHNHG** obtained for the IN selection is highlighted in bold. Statistically it corresponds to half the selected sequences. Also highlighted are amino acid motifs **H(I/L)E** and **HL(I/L)** in the second series.

*7. DNA Binding Assay*. IN was incubated under the same conditions used for the 3′-end processing and strand transfer inhibition experiments. A 1 mL sample of prewash buffer (20 mM Hepes, pH 7.6, 10 mM $MnCl_2$, 10 mM NaCl, and 0.01% BSA) was added, and the reactions were filtrated with the multifilter Millipore system, through nitrocellulose filters (0.45 $\mu$m, Whatman) initially rinsed in prewash buffer for 10 min. Then the filters were washed twice with 4 mL of wash solution (20 mM Hepes, pH 7.6, 10 mM $MnCl_2$, 30 mM NaCl) and dried. The retained radioactivity was quantified in a liquid scintillation counter (Wallac 1409).

## RESULTS AND DISCUSSION

*Selection of Phages Expressing Peptides That Bind to HIV-1 Integrase*. To search for peptides that bind specifically to the HIV-1 integrase, the phage peptide display selection method was used. This technique consists of expressing peptides fused to a coat protein (pIII) of M13 bacteriophage and displaying the fused protein on the surface of the phage so that it can interact with the target, thus allowing the screening of a large number of random short peptide sequences.

The random heptapeptide phage display library was incubated with purified recombinant IN followed by washing away the unbound material and eluting the specifically bound phages. These selected phages were then amplified and taken through additional binding/elution/amplification cycles with increasingly stringent conditions during the washing step to enrich the pool in favor of the tightest binding sequences. The enrichment rate of the recovered phages reached a plateau after 3 panning rounds, and 20 individual phages were chosen among those identified as positive clones able to bind to IN. The peptide sequence of these clones was deduced from their DNA sequence. In a parallel experiment, bovine serum albumin (BSA) was used as a control with the same library. Table 1 shows the phagotope sequences selected against IN and BSA. No common sequences were

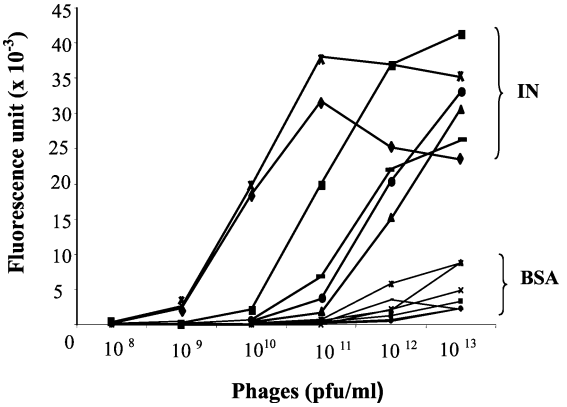


FIGURE 1: Binding of selected phages to immobilized IN. Binding of phage-displayed peptides (from $10^8$ to $10^{13}$ pfu/mL) was measured using the ELISA assay. After incubation to allow phage−IN binding, excess phages were washed off. Anti-M13 phage antibody conjugated with HRP was used to reveal bound phages after addition of peroxidase substrate. The binding of IN-selected phages to IN (100 $\mu$g/mL) was compared to that of BSA (100 $\mu$g/mL) used as a control. The data represent mean values of three independent experiments. The phage display heptamer sequences are FHNHGKQ (■), RLFTWEG (●), HIEHLIA (◆), HLEHLLF (×), STPIPAP (▲), and STFTHPR (—).

found among the peptides selected with each protein, thus strongly suggesting that the phages were selective toward their respective target.

For IN selection, 50% of the clones analyzed contained the same sequence, i.e., FHNHG. The recovery of a consensus sequence confirmed that an enrichment of bound phage clones had occurred during the successive rounds of panning. Another peptide motif, H(I/L)EHL(I/L), was also present in some of the remaining clones. All the sequences of the displayed peptides were aligned using a protein sequence databank (BlastP search in Swiss-Prot) and with protein sequences well-known to interact with IN. No significant sequence similarities were underscored, either with human or with HIV-1 known protein sequences, including HIV-1 IN.

In the case of selection with BSA, no consensus sequence was found though some identical clones were isolated. In general, the clones selected with BSA had a high percentage of hydrophobic aromatic amino acid residues. This observation can be related to the report describing the unspecific binding of phage display random library products to plastic containers (*32*). Thus, peptides rich in aromatic amino acids may preferentially bind to plastic plates by hydrophobic interactions, supporting the nonspecific peptide−BSA binding.

To further assess the selectivity of the heptapeptides toward IN, each individual clone was assayed by the ELISA method. IN- or BSA-coated wells were incubated with several concentrations of IN-selected phages. A range between $10^8$ and $10^{13}$ plaque-forming units (pfu)/mL was used, corresponding to phage concentrations of 0.16 pM to 16 nM, respectively. At these concentrations, an unambiguously positive ELISA signal can only be observed if the binding affinity is in the micromolar or lower range. Figure 1 presents the results of the binding of some representative phage-displayed peptides and shows that most phages bound markedly better to IN than to BSA. This result confirmed the specificity of the phages for their target. However, not

Table 2: Relative Affinity of Selected Phages for HIV-1 IN Deduced from the Binding Curves[a]

| phagotope sequence | $FC_{50}$[b] (pfu/mL) | phagotope sequence | $FC_{50}$[b] (pfu/mL) |
|---|---|---|---|
| **FHNHGKQ** | $10^{11}$ | **HLEHLLF** | $10^{10}$ |
| FHNHGAA | $5 \times 10^{11}$ | **HIEHLIA** | $10^{10}$ |
| FHNHGAT | $5 \times 10^{11}$ | PFFHLIG | $5 \times 10^{11}$ |
| FHNHGIL | $5 \times 10^{11}$ | STFTHPR | $5 \times 10^{11}$ |
| FHGHGLY | $7 \times 10^{11}$ | SSLPLRK | $10^{12}$ |
| FHNHGAP | $>10^{12}$ | RLFTWEG | $>10^{12}$ |
| FHNHGST | $>10^{12}$ | STPIPAP | $>10^{12}$ |

[a] Heptapeptide sequences presenting the best $FC_{50}$ values are highlighted in bold. [b] $FC_{50}$ is defined as the phage concentration that leads to 50% binding to the protein target. For each phage 100% binding was the fluorescence value reached at the plateau of the binding curves in Figure 1.

all IN-selected phages bound with the same affinity to IN. A method to quantify this interaction was to determine the relative affinity of binding by measuring their $FC_{50}$. This value was defined as the phage concentration leading to 50% binding to IN, considering that 100% binding of each phage was the fluorescence value reached at the plateau. Phages containing peptides with the HLEHLL and HIEHLI motifs were the strongest ligands with an $FC_{50}$ of $10^{10}$ pfu/mL, followed by the peptide containing the consensus motif FHNHGKQ (Table 2). Note that the last two amino acids of the FHNHG-displaying phage have a strong influence on their relative affinity toward IN.

Taken together, these results show that screening with the phage display method allowed us to identify several peptides that strongly and specifically interact with HIV-1 IN.

*The Selected Synthetic Peptides Are Able To Interact with HIV-1 IN*. The two phage-displayed peptides corresponding to sequences FHNHGKQ and HLEHLLF that showed the highest capacity for IN binding were used for further studies of the interaction with the enzyme and their potential effect on its in vitro activities. These peptides were chemically synthesized with the linker sequence GGGS at the C-terminus, present in the fusion protein at the phage surface.

The relative affinity of the two synthetic peptides was measured by their ability to compete for IN binding with their corresponding peptide-displaying phage using a competitive ELISA assay. Increasing concentrations of the peptides were incubated with coated IN in the presence of a constant amount of the selected clone. These competition assays were compared to that with a random peptide not included in those selected using the panning strategy. Its sequence (LVDSTFI) was chosen so that no amino acid was present at the same position in the selected peptides. As shown in Figure 2, both FHNHGKQ and HLEHLLF peptides were able to displace their respective phage binding to IN, while the random peptide was not able to compete in the same range of concentrations. These results further support the specific nature of the interaction between HIV-1 IN and the selected peptides.

The relative affinity was calculated as the peptide concentration leading to 50% inhibition ($IC_{50}$) of phage binding to IN. Peptide HLEHLLF presented a 10-fold higher affinity for IN than peptide FHNHGKQ with $IC_{50}$ values of 100 nM and 1 $\mu$M, respectively (Figure 2).

*Effect of Synthetic Peptides on in Vitro IN Activities*. The integration reaction can be reproduced in vitro using

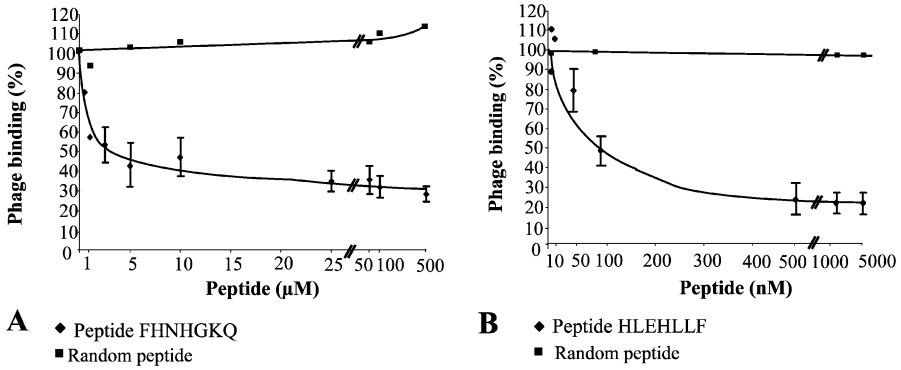


FIGURE 2: Competitive ELISA between phages and the corresponding synthetic peptides. Competitive ELISA was performed as a usual ELISA assay except that the selected phage was preincubated with the random or the corresponding synthetic peptide before addition to the IN-coated wells. Increasing concentrations of peptides were used as indicated in the figure, and a constant amount of the phage-displayed peptide corresponding to 50% binding to IN, as determined in previous ELISA tests. Competition assay with the FHNHGKQ-displaying clone (A) and HLEHLLF-displaying clone (B).

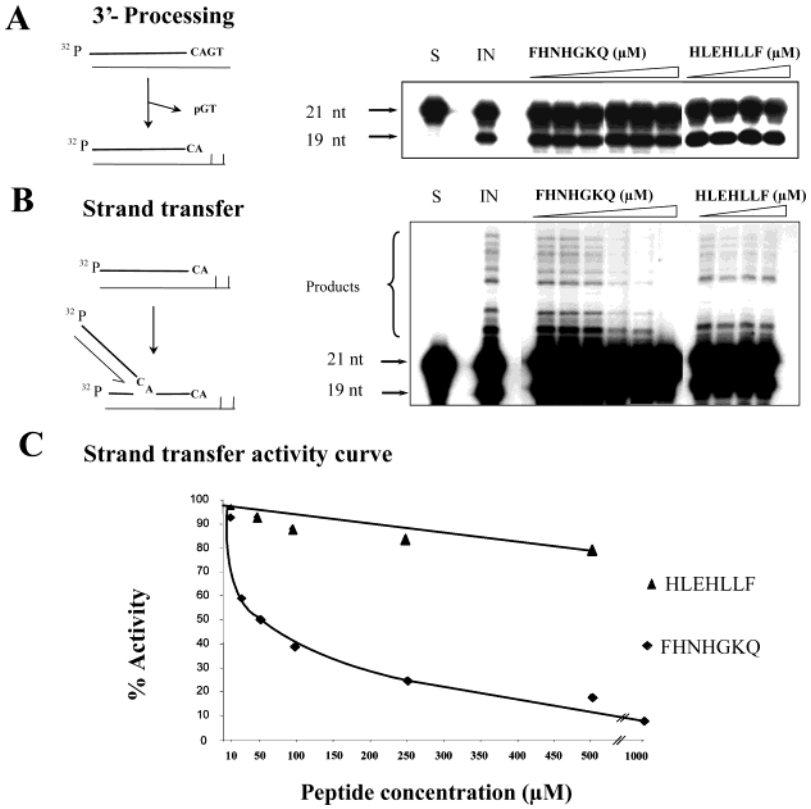


FIGURE 3: Effect of selected peptides on in vitro IN activities. (A) Left: schematic representation of the 3′-processing. Right: reaction mixtures containing 300 nM IN in the presence of increasing concentrations of each peptide were incubated at 37 °C for 20 min before addition of 10 nM substrate and incubated for an additional 15 min at 37 °C. Lane S corresponds to the substrate and lane IN to the complete reaction without peptide. Products were separated on a 12% polyacrylamide 7 M urea gel. (B) Left: schematic representation of the strand transfer reaction. Right: conditions were the same as those described for 3′-processing, except for the incubation time (30 min). Lane S corresponds to the substrate and lane IN to the complete reaction mixture without peptide. (C) Curves derived by quantifying the products obtained in the strand transfer reaction. The percentage of IN activity (%) in the presence of peptides was calculated according to the control without peptide; 100% corresponds to the activity without peptide.

recombinant IN and radiolabeled DNA substrates. Thus, the effect of the peptides was investigated on the two in vitro activities (3′-end processing and strand transfer) of HIV-1 IN by preincubating increasing concentrations of these synthetic peptides with IN. The corresponding substrates were added and reactions performed as described in the Materials and Methods.

The 3′-processing reaction corresponds to the cleavage of a dinucleotide from the 3′-end of a model DNA mimicking the U5 end of HIV-1 LTR. As shown in Figure 3A, IN catalyzed the processing of a 21-bp double-stranded ODN, giving the expected 19-mer-labeled product (lane IN). In the presence of the selected peptides, no inhibition was observed.

In the strand transfer reaction, the recessed 3′-end of a processed 21-bp double-stranded ODN becomes the attacking nucleophile for the joining reaction with a second identical ODN (Figure 3B). Integration is done in random joining sites, leading to a ladder of longer product molecules resolved on a DNA denaturing gel. Since IN can process the blunt-ended ODN in the presence of the peptides, this product can be

used to perfom the second transesterification reaction, yielding strand transfer products. We monitored the strand transfer reaction using either a preprocessed 21-pb double-stranded ODN or the full-length ODN, allowing the HIV-1 IN to catalyze the two reactions successively. The autoradiogram was overexposed to quantify the intensity of the higher molecules and to represent the graph of IN activity as a function of peptide concentrations (Figure 3C). In the presence of the peptides, a highly interesting result was obtained. A dose-dependent inhibition of the strand transfer reaction was obtained with FHNHGKQ ($IC_{50} = 70\ \mu M$). In contrast, and despite the high affinity of the HLEHLLF peptide for IN, no alteration of IN activity was observed with this compound.

These results showing a specific inhibition of the strand transfer activity of HIV-1 IN by the FHNHGKQ peptide provide strong evidence that the peptide interacts with a functional site on the integrase and not randomly with the protein surface. The inhibitory peptide might hinder either IN oligomerization or substrate binding to IN.

*Differential Effects of Peptides on IN Dimerization and Binding to Its Substrates*. To obtain further information on the inhibition mechanism, the effect of peptides on IN dimerization was tested. A cross-linking assay was performed using IN, increasing peptide concentrations, and a cross-linker agent (*cis*-aquahydroxydiamminoplatinum) as described in a previous paper (*33*). We concluded that both FHNHGKQ and HLEHLLF peptides do not disturb IN multimerization as IN monomers were still seen to diminish in favor of the dimeric form of IN when incubated with the peptides (data not shown). As IN dimerization seems necessary to perform both 3′-end processing and strand transfer reactions (*30*, *34*), it is more likely that the FHNHGKQ peptide interferes with DNA binding to IN as it inhibits only one activity.

To assess this hypothesis and better understand the mechanism implicated in the inhibition of IN strand transfer activity by FHNHGKQ, a filter binding assay was carried out to analyze DNA binding to IN in the presence of the peptides. HIV-1 IN was preincubated with increasing peptide concentrations before the addition of the radiolabeled substrates for the 3′-end processing or strand transfer reactions. No binding inhibition was obtained when the peptides were incubated with IN and the substrate for 3′-end processing (Figure 4, open symbols). Interestingly, a strong inhibition of the DNA binding to IN was observed with the FHNHGKQ peptide in the case of the strand transfer substrate, while no effect on the binding was produced with the HLEHLLF peptide (Figure 4, filled symbols). The absence of an effect on DNA binding by HLEHLLF was consistent with its lack of inhibition; its binding site most probably does not interfere with DNA binding. This assay showed that binding of the two substrates to integrase was differently affected by FHNHGKQ peptide, strongly demonstrating that this peptide inhibits strand transfer by competitively blocking only the binding of the target DNA to a specific site on IN.

Although 3′-processing and strand transfer are very similar reactions at the chemical level, the engagement of the DNA substrates in the active site domain of IN must differ in these two reactions, since for the strand transfer the active site must accommodate the target DNA in addition to the viral DNA. Recent studies support the possible existence of

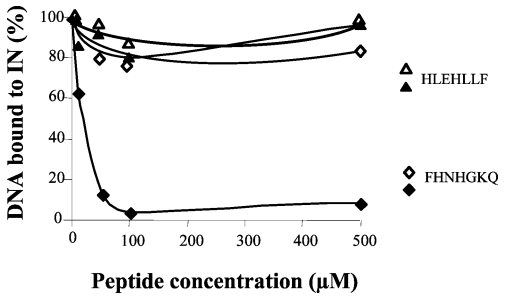


FIGURE 4: Binding of IN to DNA substrate for processing and strand transfer. IN was preincubated in the presence of different concentrations of each peptide. The reaction conditions used were the same as for the reactions using the radiolabeled ODN corresponding to processing or strand transfer reaction substrates. Following incubation, the mixtures were filtered through nitrocellulose membranes, and the radioactivity retained was quantified in a liquid scintillation counter. A value of 100% corresponds to the percentage of labeled DNA retained by IN on the filter. Open symbols correspond to processing substrate. Filled symbols correspond to strand transfer substrate.

different IN conformations or distinct sites needed in the recognition of the viral and the cellular target DNAs (*5*, *35*−*37*). In parallel, a thermodynamical model based on the SILC (stepwise increase in ligand complexity) method (*38*) also suggests an organization of the IN active site that explains specific binding of the viral and target substrates through the different steps of catalysis. The characterization of substrates binding to IN showed a relatively higher enzyme affinity for the 3′-terminal CA processed ODN than that for 3′-terminal GT nonprocessed substrate, suggesting that different subsites of IN form specific contacts with the 3′-terminal dinucleotide of viral DNA and target DNA (*38*). Furthermore, diketo acid derivatives have recently been described as selective inhibitors of HIV-1 integrase strand transfer reaction. The hypothesis of conformational changes of IN has been proposed to explain diketo acid competitive inhibition for target DNA binding (*9*−*11*, *36*). From all these studies, we can easily conceive that distinct enzyme−DNA complexes assemble during catalytic steps and that compounds such as diketo acid or FHNHGKQ peptide are able to interfere only with IN target DNA binding without affecting the interaction with viral DNA, thus leading to only strand transfer reaction inhibition. Since our peptide binds free integrase independently of the DNA substrate, its mechanism of inhibition is not likely to be the same as that of the diketo acids. So several hypotheses could be proposed to explain target DNA binding competitive inhibition by FHNHGKQ peptide: (i) the peptide could bind the same site as target DNA, considering that viral DNA and target DNA bind two distinct adjacent sites on the free IN, (ii) peptide binding near the target DNA specific site could lead to conformational changes and interfere with IN binding to its substrate, (iii) the peptide could occupy a site adjacent to the target DNA site and thus block the IN conformational changes necessary to bind target DNA but not viral DNA.

*Determination of the IN Domains Interacting with the Selected Peptides*. To gain insight into the IN domain(s) interacting with the agents selected using the phage display method, the binding affinity of peptides displayed on the phage surface was determined using different truncated forms of HIV-1 IN overexpressed in *E. coli* BL21(DE3) and purified under native conditions. The localization of the IN

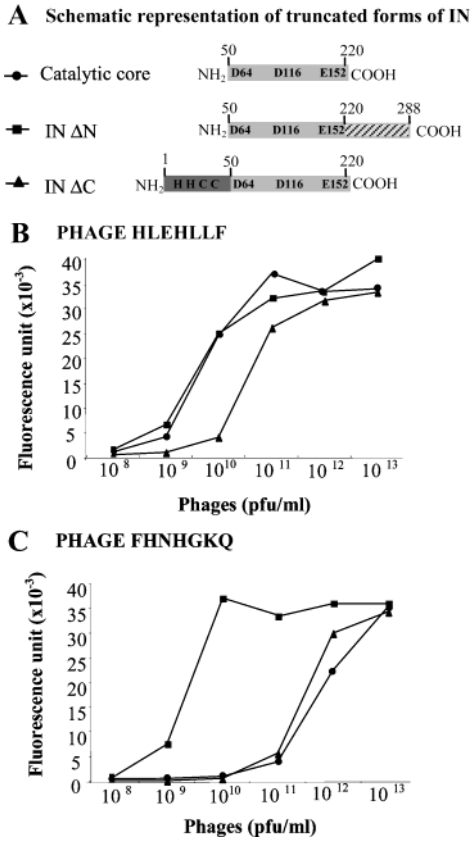


FIGURE 5: Interaction of selected peptides with different HIV-1 IN domains. The three truncated forms of HIV-1 IN shown schematically in panel A were purified as described in the Materials and Methods. Each domain was tested with the ELISA method. The coated IN (20 $\mu$g/mL) was incubated in the presence of the phage-displayed HLEHLLF peptide (B) or the FHNHGKQ peptide (C) at concentrations ranging from $10^8$ to $10^{13}$ pfu/mL.

domain interacting with the peptides was defined by ELISA, using three truncated forms of IN as targets: IN-ΔC, IN-ΔN, and the catalytic core (Figure 5A).

As shown in Figure 5B, the HLEHLLF-displaying clone interacted in a similar way with two forms of IN, i.e., IN-ΔN and the catalytic core, since the ELISA signals obtained were almost identical. However, the C-terminal-truncated IN gave a significant lower binding curve, indicating that the N-terminal domain interferes with phage binding to the catalytic core. Despite significant binding to the catalytic core domain, this peptide was not able to inhibit IN activities by affecting IN dimerization or DNA substrate binding. This is certainly due to its many potential sites of interaction on this large truncated domain which spans amino acids 50−220.

In the case of the FHNHGKQ-displaying clone, the relative affinity of the peptide was higher for IN-ΔN compared to that obtained with IN-ΔC or the catalytic core, thereby indicating that, in addition to the core domain, the C-terminal domain of IN was necessary for interaction with this peptide (Figure 5C). Since the C-terminal domain is involved in DNA binding in a non-sequence-specific manner (*6*, *7*, *39*), peptide interaction with this site might explain the selective inhibition of target DNA binding to IN and consequently the observed strand transfer inhibition (Figure 3B,C).

The crystal structure of the multidomain form of IN, comprising the catalytic core and the C-terminal domains (residues 52−288), has been elucidated (*40*), showing that a flexible elbow within helix α6 links the two domains, thus pointing to a functional flexibility of the C-terminal domain during the integration process. The authors have modeled a short DNA oligonucleotide onto an IN dimer, showing that a strip positively charged beginning at the active site of one monomer and extending along the C-terminal domain of the other monomer could be implicated in DNA binding. On the other hand, our recent mutagenesis analysis of the IN catalytic core region (*37*) showed differential substrate binding to IN depending on the type and the position of the mutated residue. A more pronounced negative effect on DNA binding was obtained with the strand transfer substrate, confirming that the binding site for target DNA could be distinct from that of the viral DNA. Taken together, the results obtained using different approaches asserted that the catalytic core and the C-terminal domain are involved in DNA binding, leading to target DNA selection. Our results are consistent with several models proposed to represent the sequential events of IN binding to viral DNA and targeting the host DNA during the catalytic steps, involving multiple interaction surfaces on the enzyme (*5*, *37*). The IN−DNA complex architecture may involve specific functions of the core and the C-terminal domains to select the integration target site. Oligomerization of the enzyme seems crucial in this process, since in the model of an active complex of at least four IN protomers, each subunit played an asymmetric role in the recognition of the different DNAs and in the calatytic reaction (*5*, *40*). However, crystallization of the entire IN and IN complexed with DNA has not yet been achieved, rendering difficult the understanding of interactions between IN and its different substrates during the multistep integration process. Finally, the peptide FHNHGKQ seems to target a specific site located on the core and C-terminal domains of free IN, leading to a competitive inhibition of target DNA binding. The precise mechanism of this specific inhibition remains to be clarified. This kind of inhibitor could be a useful tool to study the role of the IN domains implicated in DNA recognition.

## CONCLUSION

In conclusion, by using a phage display peptide library, we have identified several short peptides able to interact specifically with HIV-1 IN. One of the selected peptides (FHNHGKQ) is able to inhibit the strand transfer activity of HIV-1 IN in the absence of an effect on 3′-processing. This result suggests that the mechanisms involved in the 3′-end processing and strand transfer are not completely identical and could implicate different DNA binding sites. Indeed, although the same active site residues are required for 3′-end processing and strand transfer (*41*−*43*), the ability of our peptide to discriminate between the two catalytic functions of IN demonstrates that the enzyme possesses different structural conformations or distinct binding sites for the viral and cellular target DNAs. This model is also considered by other groups to understand IN affinity for its different substrates (*5*, *7*, *37*−*39*) and the action of selective inhibitors for the strand transfer step during the integration process (*8*−*10*, *36*). However, the structure for neither the full-length integrase nor the integrase−DNA complex has been solved. These data might help us to understand how IN and specific inhibitors work.

The HLEHLLF peptide presents a high affinity for HIV-1 IN but no inhibitory effect on its enzymatic activities. Derivatives of this peptide might be potential candidates for the in vivo inhibition of the interactions of IN−cellular proteins or IN−viral proteins. Agents of this type could be used to interfere either with the nuclear migration of the preintegration complex in infected cells or with the DNA viral integration process in the host DNA by preventing the binding of activator factors to IN. Moreover, given their affinity for the enzyme, these peptides may become useful tools to elucidate the 3D structure of the entire IN.

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