

Correlation Between Shiftide Activity and HIV-1 Integrase Inhibition by a Peptide Selected from a Combinatorial Library

Ayelet Armon-Omer¹†, Aviad Levin¹†, Zvi Hayouka², Karin Butz³, Felix Hoppe-Seyler³, Shoshana Loya⁴, Amnon Hizi⁴, Assaf Friedler^{2*} and Abraham Loyter^{1*}

¹Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

²Department of Organic Chemistry, Institute of Chemistry, The Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel

³Molecular Therapy of Virus-Associated Cancers, German Cancer Research Center, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

⁴Department of Cell and Developmental Biology, The Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

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The human immunodeficiency virus type 1 (HIV-1) integrase (IN) protein is an emerging target for the development of anti-HIV drugs. We recently described a new approach for inhibiting IN by “shiftides”—peptides that inhibit the protein by shifting its oligomerization equilibrium from the active dimer to the inactive tetramer. In this study, we used the yeast two-hybrid system with the HIV-1 IN as a bait and a combinatorial peptide aptamer library as a prey to select peptides of 20 amino acids that specifically bind IN. Five non-homologous peptides, designated as IN-1 to IN-5, were selected. ELISA studies confirmed that IN binds the free peptides. All the five peptides interact with IN with comparable affinity ($K_{d} \approx 10 \mu\text{M}$), as was revealed by fluorescence anisotropy studies. Only one peptide, IN-1, inhibited the enzymatic activity of IN *in vitro* and the HIV-1 replication in cultured cells. In correlation, fluorescence anisotropy binding experiments revealed that of the five peptides, only the inhibitory IN-1 inhibited the DNA binding of IN. Analytical gel filtration experiments revealed that only the IN-1 and not the four other peptides shifted the oligomerization equilibrium of IN towards the tetramer. Thus, the results show a distinct correlation between the ability of the selected peptides to inhibit IN activity and that to shift its oligomerization equilibrium.

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*Corresponding authors. E-mail addresses: assaf@chem.ch.huji.ac.il; loyter@mail.ls.huji.ac.il.

† A.A.-O. and A.L. authors contributed equally to the manuscript.

Abbreviations used: Y2H, yeast two-hybrid; IN, integrase; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; LEDGF, lens epithelium-derived growth factor; IBD, integrase binding domain; Gal4-BD, Gal4 binding domain; trxA, thioredoxin A; NIH, National Institutes of Health; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAGI, multinuclear activation of a galactosidase indicator; AZT, 3'-azido-3'-deoxythymidine; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline.

Introduction

The human immunodeficiency virus type 1 (HIV-1) integrase (IN) enzyme mediates the integration of the reverse-transcribed viral DNA into the host genome.¹ IN, which is composed of three functional domains, recognizes specific sequences in the long terminal repeat (LTR) elements of the viral cDNA. The integration consists of two steps: (I) 3'-end processing, where a dinucleotide is removed from the two 3'-ends of the LTR DNA (this reaction occurs in the cytoplasm, within the viral pre-integration complex), and (II) strand-transfer reaction (this takes place in the host nucleus). After entering the nucleus, the pre-processed viral double-stranded DNA is joined into the host target DNA. Due to its crucial function in the virus life cycle, inhibition of the enzymatic activity of IN should result in blocking virus infection. The fact that no cellular homologue of IN is known² makes this protein an attractive target for the development of specific anti-retroviral drugs.³⁻⁵ Recently, the first IN inhibitor was approved by the U.S. Food and Drug Administration as an anti-HIV drug.^{6,7}

The HIV-1 IN forms a stable complex with the human lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75). Binding of this cellular protein to IN is mediated by a specific domain, designated as IN binding domain (IBD).⁸ Recent work in our laboratories had focused on the development of peptides derived from LEDGF/p75 as new types of IN inhibitors. Two peptides derived from its IBD block IN enzymatic activity *in vitro*, penetrate cells and significantly inhibit HIV-1 propagation in infected cultured cells.⁹ These LEDGF/p75-derived peptides inhibited IN activity in an allosteric mechanism, by shifting the IN oligomerization equilibrium from the active dimer towards the inactive tetramer, which is unable to catalyze the first integration step of 3'-end processing.¹⁰⁻¹⁴ We termed these peptides as "shiftides,"⁹ and they serve as proof of principle for a general strategy for protein inhibition in general and a novel approach for the development of potent IN inhibitors in particular.

Inhibition of HIV-1 IN by peptides may be achieved also using other mechanisms, such as with the use of peptides that inhibit HIV-1 IN enzymatic activity by masking certain domains within the protein.¹⁵ In this work, an attempt was made to further establish the correlation between the ability of IN-interacting peptides to function as shiftides and that to block IN activity.

The selection of protein-interacting peptides under intracellular conditions was made possible by employing the yeast two-hybrid (Y2H) system combined with the screening of expression vectors coding for random peptide and peptide aptamer libraries.¹⁶ The peptide aptamers are displayed on the surface of an inert scaffold protein, and the subsequent screening of combinatorial peptide aptamer libraries may result in a peptide that can specifically interact with a given target protein

under intracellular conditions.^{17,18} Such peptides may have the potential to selectively block the function of their target protein both *in vitro* and *in vivo*. This system has successfully been used for the identification and selection of peptides that are able to interact specifically with a target protein, such as the E6 protein of human papillomaviruses.¹⁶

Previously, a peptide that interacts with HIV-1 IN was selected and demonstrated to inhibit its activity using the Y2H system.¹⁹ In this work, we screened a peptide aptamer expression library¹⁶ of high complexity ($\sim 2-3 \times 10^8$) for the ability to bind HIV-1 IN. Five peptides with no sequence homology that bound IN almost to the same extent were selected. Of these five IN-interacting peptides, only a single peptide, designated by us as IN-1, inhibited HIV-1 IN enzymatic activities *in vitro* and reduced HIV-1 infectivity in cultured cells. The inhibitory IN-1 peptide functions as a shiftide by promoting a shift in the IN oligomerization equilibrium towards the tetramer. The other four selected peptides, which bound IN but did not inhibit its activity, did not function as shiftides. Thus, it appears that binding of a peptide to IN is not sufficient by itself for IN inhibition in a shiftide mechanism.

Results

Selection of IN-interacting peptides by Y2H system

A Y2H system was used to select peptide aptamers that specifically bind IN *in vivo*.¹⁹ The complete HIV-1 IN protein fused to the Gal4 binding domain (Gal4-BD) was used as bait, and a randomized peptide aptamer expression library was employed as prey. The *Escherichia coli* thioredoxin A (trxA) protein, fused to the Gal4 transcriptional activation domain, served as a scaffold to present a random library of constrained peptides. From about 10^6 transformants, we isolated 5 clones that activated all the markers (growth in the absence of adenine, histidine and uracil). The plasmids encoding the interacting peptide aptamers were isolated from the yeast strain. As negative controls, these yeasts were transformed with a plasmid expressing Gal4-BD alone or with plasmids expressing various other unrelated proteins (LEDGF, importin α and histone H2A). No interaction with the selected peptides was detected with these control proteins (not shown).

After determining the DNA sequence of the positive plasmids, we synthesized the corresponding IN-interacting peptides, designated as IN-1 to IN-5 (for sequences, see Table 1), and estimated their ability to interact with a recombinant full-length IN *in vitro* (Fig. 1a and b). Furthermore, gel filtration experiments also showed that all these peptides indeed interacted with the IN protein, as could be inferred from the fact that the fluorescently labeled peptides were eluted together with the IN protein,

Table 1. Peptide aptamers binding to the HIV-1 IN selected using the Y2H screen

Peptide	Sequence
IN-1	WQCLTLTHRGFVLLTITVLR
IN-2	PFSNVSSLREPNLEFELVYL
IN-3	RCWLQMWQESFDLVAMLGDT
IN-4	LGTGPF AHLVLWPTRALCHA
IN-5	FVSTHFSVPASPWLLIDIV

Peptides were selected and their sequence was determined as described in Materials and Methods.

as opposed to free peptides, which were eluted in a different fraction (not shown). The selected peptides had very similar binding affinity to IN, as revealed from the K_d values measured using fluorescence anisotropy experiments (Fig. 1b and Table 2) and from the extent of binding determined using ELISA (Fig. 1a). The differences obtained in the binding affinities are not significant, since they are all at the same order of magnitude.

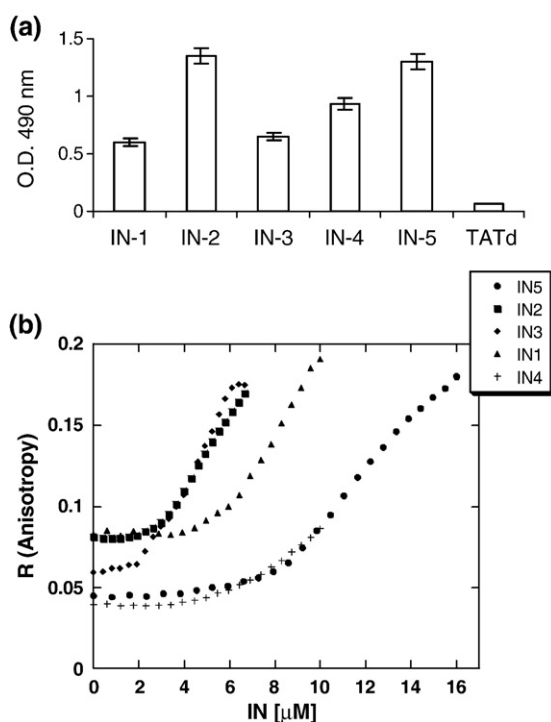


Fig. 1. Peptides selected by the Y2H system bind to IN with a similar affinity. (a) ELISA results: The five selected peptides (IN-1 to IN-5) were covalently attached to biotinylated BSA molecules as described before.²⁰ ELISA plates were coated with IN as previously described²⁰ and then incubated with 0.2 μg of each of the BSA-peptide conjugates. The degree of binding was estimated as previously described,²⁰ and the data shown are those derived after subtracting the values obtained following binding of biotinylated BSA only (equivalent to an optical density of 0.140 U at 490 nm). (b) Fluorescence anisotropy binding studies. IN was titrated into the fluorescein-labeled peptides (100 nM): (▲) IN-1, (■) IN-2, (◆) IN-3, (+) IN-4 and (●) IN-5. Data were fit to the Hill equation (see Table 2 for binding affinities).

Table 2. Binding affinity of the selected IN-binding peptides to IN

Peptide	K_d (μM)	Hill coefficient
IN-1	8.7±0.2	4.5
IN-2	5.3±0.1	4.1
IN-3	4.5±0.4	3.1
IN-4	12.5±0.7	3.6
IN-5	12.0±0.4	5.0

IN was titrated into the fluorescently labeled IN binding peptides, and binding was estimated by monitoring fluorescence anisotropy (Fig. 1b) and fitting the results to the Hill equation as described in Materials and Methods.

Only one of the selected peptides inhibits HIV-1 IN enzymatic activity *in vitro*

To study the effect of the five IN-interacting peptides on the IN enzymatic activities, we employed two *in vitro* enzymatic assay systems. The qualitative assay shows that IN-1, but not IN-2, inhibited both the 3'-end processing and strand-transfer activities of IN (Fig. 2a and b). A densitometer analysis reveals that IN inhibition by IN-1 was concentration dependent, blocking both activities almost completely at 60 μM (Fig. 2c). The apparent concentrations leading to an inhibition of 50% of the initial IN activities (IC_{50} values) were calculated to be about 12 and 40 μM for the strand-transfer and 3'-end processing activities, respectively. This difference in sensitivity to IN inhibitory peptide is not surprising since the strand transfer is inhibited more readily than the 3'-end processing as previously described.^{21,22} No significant IN inhibition was observed with IN-2, IN-3, IN-4 and IN-5 (data not shown). To confirm these results, we also assessed the IN inhibitory capacity of the five peptides using another IN enzymatic activity assay.^{23–25} Again, only IN-1 and none of the other four IN-interacting peptides inhibited IN (Fig. 3a and b, and other data not shown).

The oligomerization equilibrium of the IN is shifted only by the inhibitory IN-1

The selection of five IN-interacting peptides, of which only one (IN-1) was inhibitory, gave us the opportunity to study the question of whether a correlation exists between the ability of a given peptide to inhibit IN and that to shift its oligomerization equilibrium. Similar to the LEDGF/p75-derived peptides,⁹ the inhibitory IN-1 also promotes the formation of IN tetramers, as was inferred from the use of commercial marker bearing the same molecular weight as an IN tetramer (black curve in Fig. 4a, and other data not shown). No detectable tetramer formation was observed in analytical gel filtration experiments after incubation of IN with the non-inhibitory peptide IN-4 (blue curve in Fig. 4a). Similarly, IN-2, IN-3 and IN-5 failed to promote the formation of IN tetramers (not shown). In line with these results, fluorescence anisotropy experiments revealed that only IN-1 and not IN-2 to IN-5 inhibited the interaction between the IN and its substrate DNA (Fig. 4b, and other data not

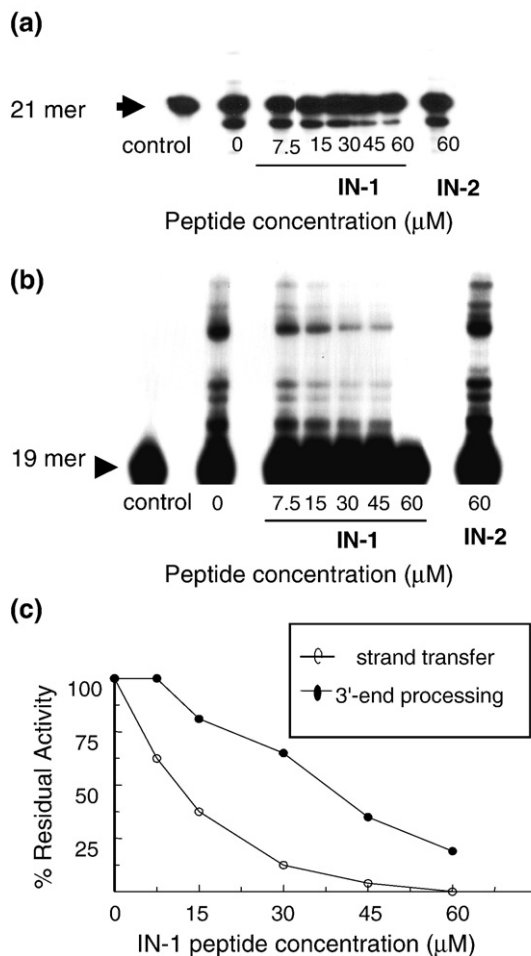


Fig. 2. Inhibition of IN activity *in vitro* by IN-1. The IN protein (187.5 nM) was incubated with peptides IN-1 and IN-2, and its 3'-end processing (a) and strand-transfer (b) activities were analyzed by the qualitative assay as described in Materials and Methods. (c) The degree of IN activity in the presence of increasing concentrations of IN-1 peptide was calculated using the densitometric software TINA (version 2.07d, Raytest Isotope Messgerate, GmbH). The IC_{50} values derived from the attached inhibition curves are 12 and 38 μ M for the strand-transfer and 3'-end processing reactions, respectively.

shown). The results depicted in Fig. 4c confirm the view that only IN-1 promotes the formation of IN tetramer when incubated with the IN protein. The IN protein was chemically cross-linked and its profile was then analyzed by SDS-gel electrophoresis to preserve the oligomeric structure obtained in the presence of the peptides. A tetrameric protein appeared following the incubation of IN only with the inhibitory IN-1 and not with the non-inhibitory IN-4 peptide (Fig. 4c).

The IN-1 peptide as well as the non-active IN peptides interact with the same amino acid sequences of the IN protein

We tested whether the same regions in IN protein bind the inhibitory and the non-inhibitory IN-interacting peptides using a peptide library spanning the full

length of the IN protein [National Institutes of Health (NIH) AIDS Research and Reference Reagent Program]. This was performed by determining the binding of bovine serum albumin (BSA) conjugates bearing the IN-1, IN-3 and IN-4 peptides to ELISA plates coated with the IN-derived peptides from the NIH library (see Materials and Methods and Table 3). As can be seen in Table 3, the active IN-1 and the non-active peptides (IN-3 and IN-4) were bound essentially to the same binding sequences derived from IN, and no specific "shifting sequence" was revealed using the ELISA binding assay system. However, it should be noted that the binding experiments were performed using IN-derived peptides and not the IN protein itself. Furthermore, the peptides represent sequences from the IN monomer and do not take into account the IN structure as a dimer or a tetramer. We assume that it is possible that in the context of oligomeric IN, there are different binding sites for the different peptides.

Cell permeability and toxicity of the IN-interacting peptides

Incubation of fluorescently labeled IN-1, IN-2, IN-4 and IN-5 peptides with cultured HeLa cells

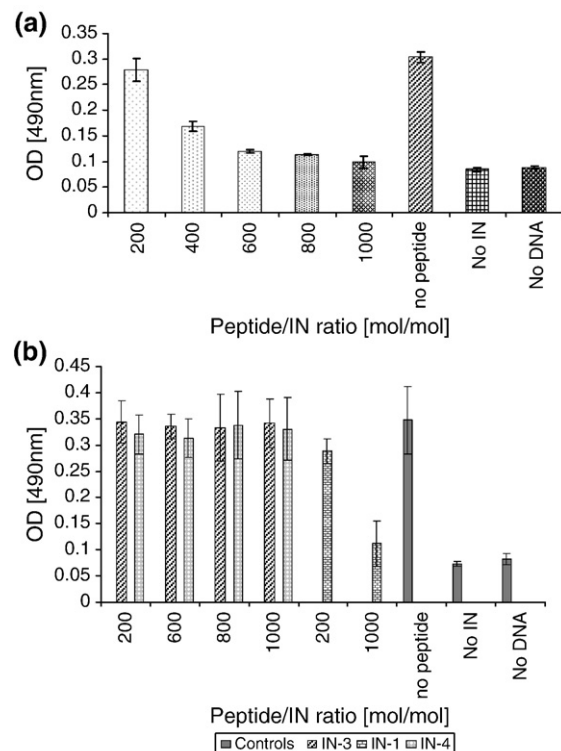
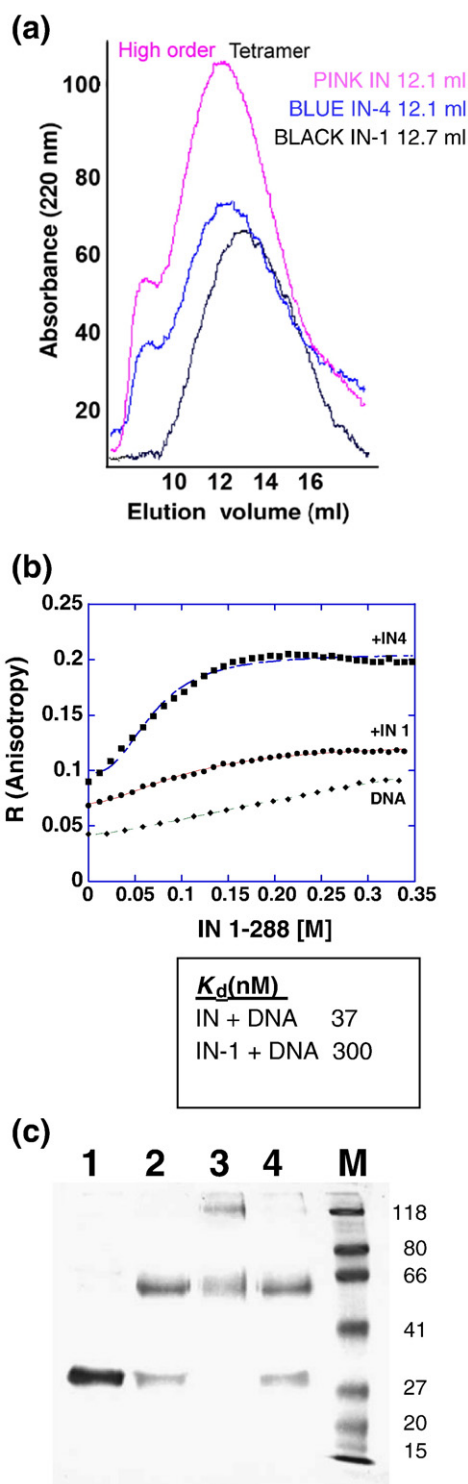


Fig. 3. The effect of IN-1 to IN-5 peptides on IN activity *in vitro*: Inhibition by IN-1. IN protein (390 nM) was incubated in the presence of IN-1, IN-3 and IN-4 peptides at the indicated peptide/IN ratios. The overall integration process was monitored using the quantitative assay system as described in Materials and Methods. (a) Inhibition of IN activity by IN-1. (b) Inhibition of IN activity by IN-1 compared with the lack of inhibition of IN-3 and IN-4.

resulted in the appearance of intracellular fluorescence, indicating their ability to translocate through the cells' plasma membrane (Fig. 5a). On the other hand, IN-3 was cell impermeable (Fig. 5a), as no intracellular fluorescence could be detected (Fig. 5a). None of the IN-interacting peptides was toxic to the HeLa as well as H9 T-lymphocyte cultured cells at the concentrations employed (up to $\sim 62.5 \mu\text{M}$), as reflected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 5b and c).



Only IN-1 and none of the other selected peptides inhibits HIV-1 infectivity in cultured cells

The ability of the IN-interacting peptides to inhibit HIV-1 replication was studied using virus-infected multinuclear activation of a galactosidase indicator (MAGI; TZM-bl) cells, whose percentage of blue cells indicates the titer of the infectious virus.²⁶ The results in Fig. 6a show that of the four peptides that penetrated cells, only IN-1 inhibited virus infection in a dose-response manner, reaching a maximum inhibition of about 75% in the presence of $62.5 \mu\text{M}$ of peptides. Inhibition of HIV-1 infectivity was also observed with IN-1 when HIV-1 infection was monitored by the appearance of the p24 HIV-1 protein (Fig. 6b) and from the level of viral DNA integrated into the cellular genome (Fig. 6c). Neither IN-1 nor the other IN-interacting peptides inhibited the synthesis of viral cDNA, as was estimated after the first cycle of infection, 6 h post-infection (Fig. 6d, and other data not shown). This strongly suggests that the inhibition of viral infectivity by peptide IN-1 results from the intracellular inhibition of the integration process, since no other step prior to integration seems to be affected. As expected, inhibition of viral DNA synthesis was observed only after incubation of the virus-infected cells with the potent reverse transcriptase inhibitor 3'-azido-3'-deoxythymidine (AZT)²⁷ (Fig. 6d). These results further indicate that IN-1 inhibited the integration step of the virus infection cycle in cells.

Inhibition of the integration step can also be inferred from Fig. 7, which summarizes the results obtained following the addition of various HIV-1 inhibitory compounds to virus-infected cells at different times.²⁸ As expected, no effect on virus infectivity, as estimated by the appearance of the p24 level, was observed following the addition of IN-4 at

Fig. 4. Effect of the IN-binding peptides on the oligomeric state of IN: Tetramerization by IN-1. (a) IN ($14 \mu\text{M}$) alone (pink) or in the presence of $14 \mu\text{M}$ IN-1 (black) or IN-4 (blue) was analyzed by analytical gel filtration as described in Materials and Methods. Only IN-1 and not the other peptides shifted the oligomerization equilibrium of IN towards the tetramer (black). (b) Effect of IN-interacting peptides on the DNA binding of IN fluorescence anisotropy studies. IN protein was titrated into fluorescein-labeled HIV-1 LTR DNA (10 nM) alone (\blacklozenge) or in the presence of $1 \mu\text{M}$ of (\bullet) IN-1 or (\blacksquare) IN-4 as described in Materials and Methods. (c) The IN protein was incubated with the IN-1 and IN-4 peptides and then cross-linked using bis(sulfosuccinimidyl)suberate, and the preparations obtained were analyzed by 10% SDS-gel electrophoresis. All other experimental conditions are as those described in Materials and Methods. Lane 1, non-cross-linked IN; only a monomeric state can be observed. Lane 2, cross-linked IN; please note the appearance of monomeric and dimeric states. Lane 3, the IN protein was incubated with IN-1 and following cross-linking was analyzed; as can be seen, the monomeric state disappears and a tetramer appears in addition to the dimer. Lane 4, the same as lane 3 but in the presence of IN-4.

Table 3. The interaction between IN-1, IN-3 and IN-4 with peptides obtained from a library covering the full length of the IN protein

Peptide number ^a	Sequence ^b	IN amino acid residues	Interacts with IN-1	Interacts with IN-3	Interacts with IN-4
5656	THLEGGKIILVAVHVA	66–80			+
5657	GKIILVAVHVASGYI	70–84	+		+
5663	GQETAYFLLKLAGRW	94–108	+		+
5664	AYFLLKLAGRWPVKT	98–112	+	+	+
5669	GSNFTSTTVKAACWW	118–132	+	+	+
5670	TSTTVKAACWWAGIK	122–136			+
5671	VKAACWWAGIKQEFQ	126–140			+
5682	EHLKTAVQMAVFIHN	170–184			+
5692	TKELQKQITKIQNFR	210–224			+
5693	QKQITKIQNFRVYYR	214–228		+	+
5699	PAKLLWKGEAVVIQ	238–252			+
5700	LWKGEAVVIQDNDS	242–256	+		
5701	EGAVVIQDNDSIKVV	246–260		+	

A peptide library spanning the full length of the HIV-1 IN subtype B consensus sequence was screened for interactions with IN-1, IN-3 and IN-4. The peptide library, obtained from the NIH AIDS Research and Reference Reagent Program, contains 73 peptides, each consisting of 15 amino acids in length, with an 11-amino-acid overlap between sequential peptides. ELISA plates were coated with IN-derived peptides of the library as previously described.²⁵ The IN-1, IN-3 and IN-4 peptides were covalently attached to BSA molecules,²⁵ and the binding of the resulting conjugates to the surface bound IN-derived peptides was estimated, using the ELISA assay system, essentially as described before²⁵ and in Materials and Methods.

^a As designed by the NIH AIDS Research and Reference Reagent Program [<http://www.aidsreagent.org>].

^b From the HIV-1 Consensus B Pol sequence [<https://www.aidsreagent.org/reagentdetail.cfm?t=peptides&cid=197>].

different intervals after infection. Dextran sulfate, a polyanion that interferes with virus–cell interaction,^{29–31} blocked virus infection only when added at time zero and was completely inactive when added 4 h after virus infection. On the other hand, as expected, the reverse transcriptase inhibitor AZT²⁷ gradually lost its inhibitory effect when added between 4 and 10 h post-infection and finally became completely inactive after 12 h. The kinetics shown in this figure clearly suggests that the peptide IN-1 inhibits a retroviral step that follows the reverse transcription step, thus confirming the results shown in Fig. 6d that the intracellular integration step is inhibited. IN-1 was still inhibitory even when administrated 12 h post-infection, at the time point at which AZT was completely inactive (Fig. 7). Nevertheless, the results in Fig. 7 confirm those shown in Fig. 6b, demonstrating that AZT was a stronger inhibitor than IN-1.

Discussion

In the current work, we have described the selection and identification of a new peptide that binds and inhibits HIV-1 IN *in vitro* and, consequently, HIV-1 infection in cultured cells. Peptides that block HIV-1 replication by inhibiting IN activity have been obtained and described before.^{9,19,21,22,25} For example, peptides that bear the α 1- and α 5-helices of IN (residues 93–107 and 167–187, respectively) strongly inhibited the dimerization of IN protein and, consequently, its enzymatic activity.³² It has been well established that the IN catalytic domain dimerizes and that this dimerization is required for the first step of the integration process, namely, the 3'-end processing of the viral DNA.^{11–14} Li *et al.*³³ reported the synthesis of two peptides, NL-6 and NL-9, with a capacity to inhibit the IN

activity at IC₅₀ values of 2.7 and 56 μ M, respectively. These peptides were designed to contain at least one amino acid residue that is important for IN catalytic activities (3'-end processing and strand transfer) or viral replication.³³ The authors suggested that it is possible that these peptides interfere with IN–DNA binding or with IN multimerization but did not present data to support this claim.³³ The Y2H system was also used in the past to select an IN inhibitory peptide by screening a yeast cDNA library.¹⁹ The selected inhibitory peptide, similar to the results reported in this work, was able to block the IN enzymatic activity *in vitro* and HIV-1 replication in cultured cells. The inhibitory activities of the peptide were attributed to its ability to disrupt the IN–viral DNA interaction.¹⁹

Recently, we described the identification of four IN-binding peptides, two of which were derived from the IBD of the LEDGF/p75 protein⁹ and the other two being from the HIV-1 Rev protein. The last two peptides were obtained based on a specific interaction between the HIV-1 IN and Rev proteins as was observed by us.²⁵ All the four peptides blocked IN enzymatic activity and HIV-1 replication in cultured cells.^{9,25}

It appears that all these peptides, including IN-1, are not toxic at the concentrations that cause inhibition of HIV-1 replication.^{9,25} Comparing the ability of the various peptides to inhibit HIV-1 replication in cultured cells, it appears that LEDGF/p75- and Rev-derived peptides are a bit more efficient as inhibitors in cultured cells.^{9,25} However, it is too early at this stage to decide which peptide will serve as the best lead compound for further development of an anti-HIV-1 drug. This will depend very much not only on their inhibitory efficiency but also on their half-life time within the circulation of lab animals, namely, their metabolic stability. It appears that all the inhibitory peptides,

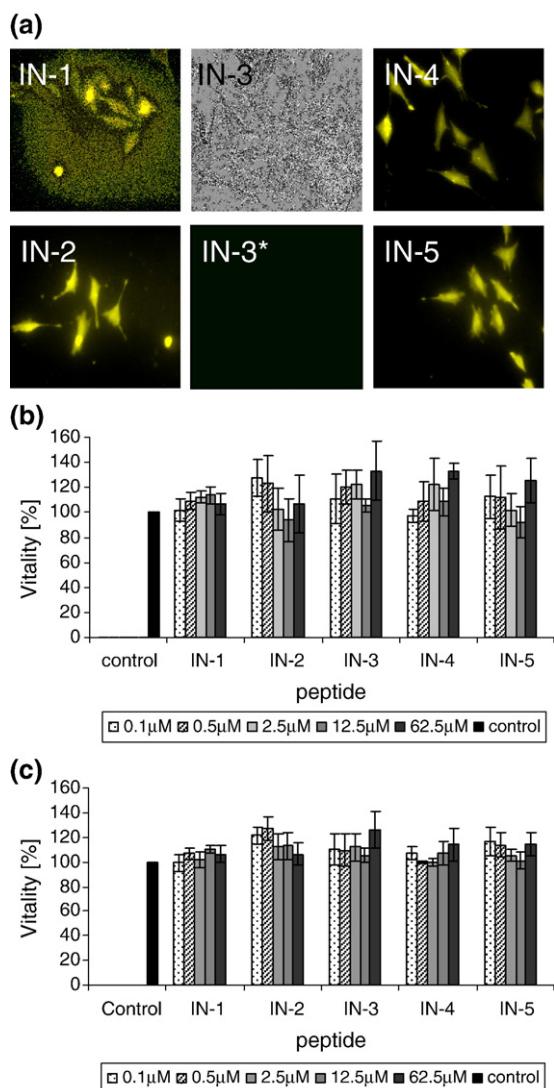


Fig. 5. Cell penetration and toxicity of the IN peptides. (a) Fluorescently labeled peptides (10 μ M) were incubated for 1 h in 37 $^{\circ}$ C with HeLa cells as described previously²⁵ and in Materials and Methods. The cells were then washed with PBS and visualized by a fluorescent microscope. No intracellular fluorescence was seen following incubation with the IN-3 peptide. IN-3 and IN-3* are pictures taken from the same field following observation by phase contrast and fluorescence microscopy, respectively, in order to demonstrate that the lack of fluorescence is not due to the absence of cells in the microscopic field. HeLa (b) and H9 T-lymphocyte (c) cell toxicities were determined by the MTT assay as described before²⁵ and in Materials and Methods.

including IN-1, inhibit HIV-1 replication by the same mechanism. This should be inferred from the results showing that the inhibition degree obtained by a mixture of IN-1 with various inhibitory peptides is not synergistic but an average of the inhibition degrees obtained by each of the peptides in the mixture (Fig. 8).

Detailed studies revealed that the LEDGF/p75 peptides inhibited the binding of the IN to DNA molecules by shifting the oligomerization state of

the IN from dimer to tetramer.⁹ IN tetramers—as opposed to dimers—are unable to execute the 3'-end processing step.¹⁴ This is in contrast to the previously described derived IN inhibitory peptides that were suggested to alter the IN oligomerization state from dimers to monomers by competitive inhibition mechanism.³² Due to their ability to interact specifically with the IN tetramer and consequently shift its oligomerization equilibrium, we termed the LEDGF/p75-derived peptides as “shiftides.”⁹

In this study, we selected IN inhibitory and non-inhibitory peptides from the combinatorial libraries using the Y2H system, all of which bind IN almost to the same degree. Our screening for IN-binding peptides resulted in five peptides, of which only one was inhibitory. This allowed us to study the question of whether a correlation exists between the inhibitory activity of the IN-interacting peptides and their ability to act as shiftides. In principle, inhibition of the IN activity by a given peptide may be attributed to either the ability of the IN-interacting peptides to act as a shiftide or that to disrupt directly the IN enzymatic activity. Our results show that of the five selected IN-binding peptides, only the inhibitory IN-1 acted as a shiftide and was able to promote the formation of IN tetramers. Although all the other four selected peptides interacted with IN almost with the same affinity, they failed to shift its oligomerization state and were not inhibitory. Thus, it is our view that a clear correlation was established in this case between the shiftide and the inhibitory features of the selected IN-interacting peptides.

The possibility that also the previously described IN inhibitory peptides and especially those that blocked IN–DNA interaction¹⁹ exert their activity by the same shiftide mechanism cannot be ruled out. In principle, shiftides act by binding preferentially to one side of the oligomerization equilibrium, and all peptides that act as IN shiftides should bind tighter to the tetramer than to the dimer of IN. It is necessary to reveal the peptide binding sites in the IN protein at its dimeric and tetrameric forms. From our binding experiments using ELISA assay systems and BSA conjugated peptides,³⁴ it appears that the inhibitory IN-1 as well as the non-inhibitory peptides IN-2 to IN-5 interact almost with the same amino acid sequences of monomeric IN. This is not surprising, since the basis of the shiftide mechanism is selective binding towards one oligomeric state, and should be studied in the context of the oligomeric proteins in order to get a clear mechanistic and structural basis for the binding selectivity. Such structural and biophysical studies as NMR and x-ray crystallography analysis with the intact homooligomeric IN protein are currently being conducted in our laboratory to obtain a more clear view of the “shifting mechanism.”

This article describes a new peptide that blocks HIV-1 replication in cultured cells due to its ability to inhibit the viral IN activity. The inhibitory IN-1 may serve as a lead compound for the development of a new family of anti-HIV drugs. In addition, a clear correlation between the inhibitory effect of the IN-1

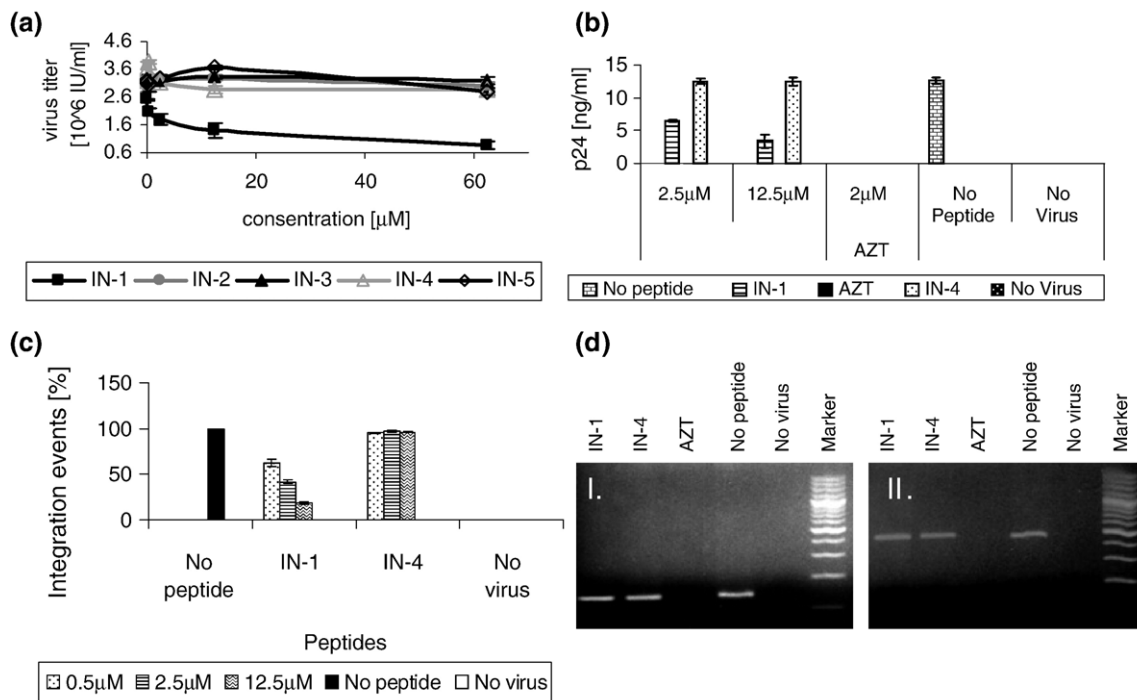


Fig. 6. Inhibition of HIV-1 replication by IN-1. (a) TZM-bl cells were incubated with the indicated peptides at the indicated concentrations; following HIV-1 infection, β -galactosidase was activated by the HIV-1-expressed Tat protein.^{25,26} (b) T-lymphoid H9 cells were incubated with the indicated peptides; after infection with HIV-1, p24 content was estimated. (c) SupT1 T-lymphoid cells were incubated with the indicated peptides at the designated concentrations; following HIV-1 infection, the percentage of integrated viral DNA was assessed. (d) Effect of IN-binding peptides on total viral DNA in HIV-1-infected cells was tested in SupT1 cells, which were incubated with 12.5 μ M of the indicated peptides or with 2 μ M of AZT for 2 h; following HIV-1 infection and 6 h of incubation, the viral Gag (I) or Nef (II) DNA sequences were amplified using specific primers. All other experimental details are as those described in Materials and Methods.

peptide and its ability to act as a shiftide, namely, to shift the oligomerization equilibrium of IN towards the inactive tetramer, has been demonstrated in this work.

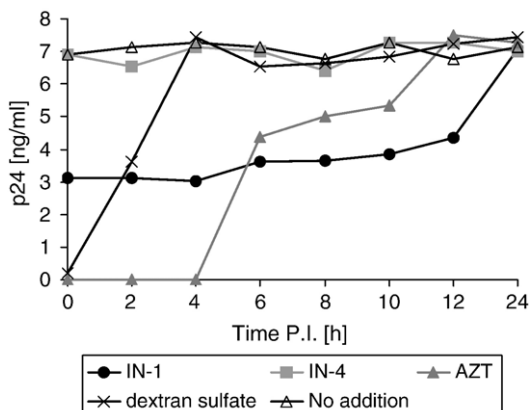


Fig. 7. Inhibition of HIV-1 replication by IN-1 is dependent on its time of addition. SupT1 cells were infected with HIV-1 at an m.o.i. of 2, and the test compounds were added at different time points after infection (0, 2, 4, 12 and 24 h). Viral p24 Ag production was determined at 48 h post-infection: (Δ) control, (\times) dextran sulfate at 20 μ M, (\blacktriangle) AZT at 2 μ M, (\bullet) IN-1 at 12.5 μ M and (\blacksquare) IN-4 at 12.5 μ M.

Materials and Methods

Cells

Yeast cells

Yeast strain KF1 (*MAT α trp1-901 leu2-3,112 his3-200 gal4 Δ gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ SPAL10-URA3*)¹⁶ was used for the peptide screening and grown in YPD medium [1% (w/v) yeast extract, 1% (w/v) tryptone and 2% (w/v) glucose]. Selective medium [synthetic dropout; 0.67% (w/v) yeast nitrogen base without amino acids and 2% glucose] was used, where tryptophan (200 mg/l), leucine (1 g/l), adenine (1 mg/l), histidine (200 mg/l) and uracil (200 mg/l) were added as required.

Mammalian cultured cells

Monolayer adherent HeLa cells, HEK293T cells and HeLa MAGI (TZM-bl) cells,³⁵ expressing the β -galactosidase gene under regulation of the transactivation response element,²⁶ were grown in Dulbecco's modified Eagle's medium. The T-lymphocyte cell lines SupT1 and H9 were grown in RPMI 1640 medium; all cells were provided by the NIH AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). Cells were incubated at 37 $^{\circ}$ C in 5% CO₂ atmosphere. All media were

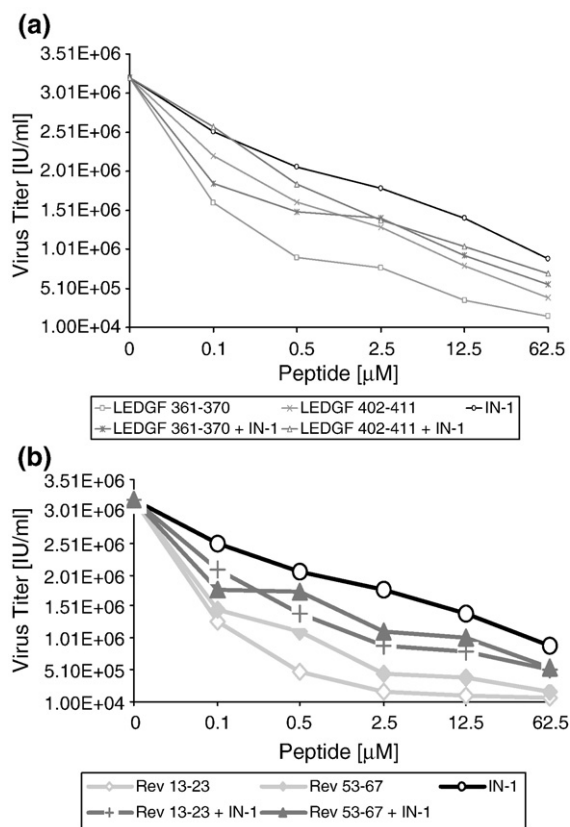


Fig. 8. Inhibition of HIV-1 replication by mixtures containing IN-1. TZM-bl cells were incubated with the indicated peptide mixtures at the indicated concentrations and following HIV-1 infection were tested for β -galactosidase activity.^{25,26} (a) Mixtures of IN-1 with LEDGF-derived peptides. (b) Mixtures of IN-1 with Rev-derived peptides.

supplemented with 10% (v/v) fetal calf serum, 0.3 g/l of L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (Biological Industries, Beit Haemek, Israel).

Peptide aptamer screening by the Y2H system

Peptide aptamer screening was performed essentially as previously described.¹⁶ The HIV-1 full-length IN hybrid expression plasmid pPC97-IN (bait plasmid) was constructed by fusing HIV-1 IN to the Gal4-BD. This was performed by cleaving a DNA fragment encoding the IN sequence from the pYES2yEGFP-IN³⁶ with SalI and BamHI and inserted into the (compatible cohesive ends with the BamHI) SalI- and BglII-digested pPC97 vector. The yeast prey vector pADTRX encodes the *E. coli* trxA gene fused to the Gal4 transcriptional activation domain. A 20-amino-acid randomized peptide library was inserted into the RsrII site of trxA, which corresponds to a constrained loop, and this library contains 2×10^8 peptide aptamers.¹⁶ The yeast strain KF1 was transformed by bait (bearing the HIV-1 IN) and then by prey (bearing the peptide aptamer library) plasmids using the lithium acetate method.³⁷ KF1 transformants expressing pPC97-IN and the peptide aptamer expression library were selected initially for growth in the absence of adenine on agar plates containing a synthetic dropout medium

lacking tryptophan, leucine and adenine. Subsequently, positive clones were transferred into replicas plated on histidine- and uracil-deficient media to analyze their ability to induce the GAL1-HIS3 and SPO13-URA3 markers of KF1. Growth under screening conditions occurs only if a peptide binds to the IN, bringing the activation domain in the vicinity of the binding domain, thereby creating a transcription factor that activates the expression of a selectable marker. The plasmids, encoding the IN-interacting peptide aptamers, were recovered using the lyticase protocol and isolated from the yeast strain colonies. Recovered plasmids were transferred into *E. coli* DH5 α for isolation, and the DNA sequence of the insert was determined. Finally, the recovered plasmids were retransformed into the yeast bait strains to confirm specific interaction.

Peptide synthesis, labeling and purification

Peptides were synthesized on an Applied Biosystems (ABI) 433A peptide synthesizer. When needed, the peptides were also labeled with tryptophan at their N-terminus for UV spectroscopy. The peptides were labeled using 5'- and 6'-carboxyfluoresceinester (Molecular Probes) at the N-terminus for the fluorescence anisotropy and cellular uptake studies.⁹ The peptides were purified on a Gilson HPLC using a reversed-phase C8 semi-preparative column (ACE) with a gradient from 5% to 60% acetonitrile in water [both containing 0.001% (v/v) trifluoroacetic acid]. Peptide concentrations were determined using a UV spectrophotometer (Shimadzu) as previously described.³⁸

Viruses

Wild-type HIV-1 and Δ env/VSV-G pseudotyped virus were generated by transfection of HEK293T cells with pSVC21 plasmid containing the full-length HIV-1_{HXB2} viral DNA.³⁹ The wild-type and Δ env/VSV-G viruses were harvested from HEK293T cells 48 and 72 h post-transfection with pSVC21 Δ env. The viruses were stored at -75°C .

Infection of cultured cells with HIV-1

Cultured lymphocytes (1×10^5) were centrifuged for 5 min at 2000 rpm; after removal of the supernatant, the cells were resuspended in 0.2–0.5 ml of RPMI 1640 medium containing virus at multiplicities of infection (m.o.i.s) of 0.1 and 2. Following adsorption for 1 h at 37°C , the cells were washed to remove unbound virus and then incubated at the same temperature for an additional 1–10 days.²⁵

HIV-1 titration by using MAGI assay

Quantitative titration of HIV-1 was carried out by the MAGI assay, as described by Kimpton and Emerman.²⁶ Briefly, TZM-bl cells were grown in 96-well plates at 10×10^3 cells per well; following 12 h of incubation at 37°C , peptides were added, and after an additional 2 h of incubation, the cells were infected with 50 μl of serially diluted HIV-1 Δ env/VSV-G virus as previously described.²⁶ The Δ env/VSV-G virus was used since for the MAGI assay only one cycle of infection is required.²⁶ Two days post-infection, cultured cells were fixed and β -galactosidase was estimated exactly as described

before.²⁶ Blue cells were counted under a light microscope at a magnification of 200 \times .

Quantitative estimation of HIV-1 infection by determination of p24

H9 lymphoid cells were incubated with the indicated peptides (see Fig. 6b) for 2 h; following infection with wild-type HIV-1 at an m.o.i. of 0.1 (as described above), the cells were incubated for 10 days. The amount of p24 was estimated in the cells' supernatant, following centrifugation for 5 min at 2000 rpm, by using an HIV-1 p24 antigen capture assay kit (SAIC, AIDS Vaccine Program, Frederic, MD), in accordance to the standards and instructions supplied by the manufacturers. Cells were counted prior to the centrifugation step; the differences between numbers of cells never exceeded $\pm 10\%$.

Protein expression and purification

The histidine-tagged IN expression vector was a generous gift of Dr. A. Engelman, and its expression and purification were performed essentially as described previously.⁴⁰

ELISA-based binding assays

Protein-peptide binding was estimated using an ELISA-based binding assay exactly as described previously.³⁴ Briefly, Maxisorp plates (Nunc) were incubated at room temperature for 2 h with 200 μ l of 10 μ g/ml of a synthetic peptide in carbonate buffer or with the IN protein. After incubation, the solution was removed, the plates were washed three times with phosphate-buffered saline (PBS) and 200 μ l of 5% BSA (Sigma) in PBS (w/v) was added for 2 h at room temperature. After rewashing with PBS, biotinylated BSA-peptide conjugates were dissolved in PBS containing 5% BSA to give the appropriate concentrations, and the resulting solutions were further incubated for 1 h at room temperature. Following three washes with PBS, the concentration of bound biotinylated molecules was estimated after the addition of streptavidin-horseradish peroxidase conjugate (Sigma). The enzymatic activity of horseradish peroxidase was estimated by monitoring the optical density at 490 nm of the product using an ELISA plate reader (Tecan Sunrise).

Cell penetration experiments

Fluorescein-labeled peptides at a final concentration of 10 μ M in PBS were incubated with HeLa cells for 1 h at 37 $^{\circ}$ C. After three washes in PBS, non-fixed cells were visualized by a fluorescent microscope as previously described.²⁵

Determination of IN activity

We employed two IN assay systems. In the first system, the 3'-end processing and strand-transfer activities of the IN, as well as preparation of the recombinant HIV-1 IN and the gel-purified oligonucleotides used for these enzymatic assays, were performed exactly as previously described in detail.^{22,41}

Determination of the IN enzymatic activity by the second assay was performed using a previously described assay system.^{23,24} Briefly, an oligonucleotide substrate of which one oligo (5'-ACTGCTAGAGATTTCCACACTGACTA-

AAAGGGTC) was labeled with biotin on the 3'-end and the other oligo (5'-GACCCCTTTAGTCAGTGTGGAAAATCTCTAGCAGT) was labeled with digoxigenin at the 5'-end was used. When peptide inhibition was studied, the IN was pre-incubated with the peptide for 10 min prior to addition of the DNA substrate. Overall, IN reaction was followed by an immunosorbent assay on avidin-coated plates as previously described.^{24,25}

Fluorescence anisotropy

Measurements were performed at 10 $^{\circ}$ C using a Perkin Elmer LS-55 luminescence spectrofluorimeter equipped with a Hamilton microlab 500 dispenser⁴² essentially as previously described.⁹ The fluorescein-labeled peptide or DNA (1 ml, 0.05–0.1 μ M in 20 mM Tris buffer, pH 7.4, and 185 mM NaCl) was placed in a cuvette, and the non-labeled IN protein (200 μ l, ~ 100 μ M) was added in 20 aliquots of 10 μ l at 1-min intervals. The total fluorescence and anisotropy were measured after each addition using an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Data were fit to the Hill equation:

$$R = R_0 + \frac{\Delta R * (K_a^{n*} [IN]^n)}{1 + K_a^{n*} [IN]^n}$$

where R is the measured anisotropy; ΔR , the amplitude of the anisotropy change from R_0 (free peptide) to peptide in complex; $[IN]$, the added concentration of IN; and K_a , the association constant.

Quantitation of integrated HIV-1 DNA in the cellular genome

The integration reaction was estimated essentially as described before.²⁵ Briefly, following incubation of the indicated peptides with SupT1 cells for 2 h, the cells were infected with an HIV-1 Δ env/VSV-G virus at an m.o.i. of 2 (as described above) for 24 h. Integrated HIV-1 sequences were amplified by two PCR replication steps using the HIV-1 LTR-specific primer (LTR-TAG-F 5'-ATGCCACGTAAGCGAAACTCTGGCTAACTAGG-GAACCCTAG-3') and Alu-targeting primers (first-Alu-F 5'-AGCCTCCCGAGTAGCTGGGA-3' and first-Alu-R 5'-TTACAGGCATGAGCCACCG-3').⁴³

During the second-round PCR, the first-round PCR product could be specifically amplified by using the tag-specific primer (tag-F 5'-ATGCCACGTAAGCGAAACTC-3') and the LTR primer (LTR-R 5'-AGGCAAGCTTTATT-GAGGCTTAAG-3') designed by Primer Express (Applied Biosystems) using default settings. For generating a linear curve, the SVC21 plasmid containing the full-length HIV-1_{HXB2} viral DNA was used as a template. In the first-round PCR, the LTR-TAG-F and LTR-R primers were used; the second-round PCR was performed using the tag-F and LTR-R primers. The standard linear curve was in the range of 5 ng to 0.25 fg ($R=0.99$). DNA samples were assayed with quadruplets of each sample. Further experimental details are as those previously described.²⁵ The cell equivalents in the sample DNA were calculated based on the amplification of the 18S gene by real-time PCR.

PCR analysis of early viral genes

SupT1 cells were incubated with 12.5 μ M of peptides or with 2 μ M of AZT for 2 h, followed by infection with an HIV-1 Δ env/VSV-G virus at an m.o.i. of 2 and incubation

for 6 h. The viral Gag or Nef DNA sequences were amplified using specific primers as described before.²⁵ Briefly, 10 ng of the total cell DNA in a 25- μ l reaction mixture containing 1 \times PCR buffer, 3.5 mM MgCl₂, 200 μ M deoxyribonucleotide triphosphates, 300 nM primers and 0.025 U/ μ l of *Taq* polymerase. The PCR conditions were as follows: a DNA denaturation and polymerase activation step of 5 min at 95 °C and then 29 cycles of amplification (95 °C for 45 s, 60 °C for 30 s and 72 °C for 45 s).

Effect of peptides on cell viability using the MTT assay

Following incubation with the indicated peptides, the medium was removed and the cells were incubated in Earl's solution containing 0.3 mg/ml of MTT for 1 h. Subsequently, the solution was removed and the cells were dissolved in 100 μ l of dimethyl sulfoxide for 10 min at room temperature. The dimethyl sulfoxide-solubilized cells were transferred to 96-well ELISA plates, and optical density values were monitored at a wavelength of 570 nm.

Time-of-addition assay

SupT1 cells were infected with wild-type HIV-1 at an m.o.i. of 1, and the test compounds were added at different time points after infection (0, 2, 4, 12, 16 and 24 h). Viral p24 Ag production was determined at 48 h post-infection. Dextran sulfate was tested at 20 μ M, AZT was at 2 μ M and IN-1 as well as IN-4 were at 12.5 μ M.²⁸

Analytical gel filtration

Analytical gel filtration of IN (10 μ M) was performed on an AKTA Explorer using a Superose 12 analytical column 30 \times 1 cm (GE Healthcare-Pharmacia Corp.) equilibrated with a buffer (20 mM Tris, pH 7.4, 1 M NaCl and 10% glycerol), exactly as described before.^{9,25}

HIV-1 IN cross-linking assay

The HIV-1 IN (final concentration, 1.2 μ M) was incubated with varying amounts of peptides at 37 °C for 1 h in a buffer (pH 7.5) containing 25 mM Hepes, 1 M NaCl, 1 mM DTT and 1 mM ethylenediaminetetraacetic acid. The reaction mixtures were treated with the cross-linking agent bis(sulfosuccinimidyl)suberate (25 eq, Pierce) for 30 min at 37 °C. The reaction mixtures were denatured and analyzed by SDS-PAGE on 10% polyacrylamide gels.¹⁵

All the results described in this work are an average of at least triplicate determinations, where the standard deviation never exceeded \pm 20%.

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