Peptides Derived From HIV-1 Rev Inhibit HIV-1 Integrase in a Shiftide Mechanism

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ABSTRACT:

The HIV-1 Integrase protein (IN) mediates the integration of the viral cDNA into the host genome. IN is an emerging target for anti-HIV drug design, and the first IN-inhibitor was recently approved by the FDA. We have developed a new approach for inhibiting IN by "shiftides": peptides derived from its cellular binding protein LEDGF/p75 that inhibit IN by shifting its oligomerization equilibrium from the active dimer to an inactive tetramer. In addition, we described two peptides derived from the HIV-1 Rev protein that interact with IN and inhibit its activity in vitro and in cells. In the current study, we show that the Rev-derived peptides also act as shiftides. Analytical gel filtration and cross-linking experiments showed that IN was dimeric when bound to the viral DNA, but tetrameric in the presence of the Rev-derived peptides. Fluorescence anisotropy studies revealed that the Rev-derived peptides inhibited the DNA binding of IN. The Rev-derived peptides inhibited IN catalytic activity in vitro in a concentration-dependent manner. Inhibition was much more significant when the peptides were added to free IN before it bound the viral DNA than when the peptides were added to a preformed IN-DNA complex. This confirms that the inhibition is due to the ability of the peptides to shift the oligomerization equilibrium of the free IN toward a tetramer that binds much weaker to the viral DNA. We conclude that protein–protein interactions of IN may serve as a general valuable source for shiftide design. © *2008 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 481–487, 2008.*

Keywords: oligomerization equilibrium; HIV 1 integrase; peptides; HIV 1 Rev; drug design

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INTRODUCTION

The Human Immunodeficiency Virus type 1 (HIV-1) integrase protein (IN) catalyzes the integration of the viral DNA into the host genome, a crucial step in the HIV-1 life cycle.[1] IN is an emerging attractive target for the development of anti-HIV drugs, mainly because mammalian cells do not have homologous enzymes. The first anti-HIV drug that acts by inhibiting IN, Raltegravir, or MK-0518 (Merck), was recently approved by the FDA.[2–7] Another IN inhibitor, GS-9137 (Gilead), is currently in phase III in clinical trials.[3] The integration proceeds by two steps[8]: (i) 3′-end processing, in which IN creates the DNA template for integration by removing dinucleotides from the 3′ ends of both LTR termini of the viral cDNA. This

step is carried out in the cytoplasm by two IN dimers that bind the viral DNA in its two LTR termini[9]; (ii) Strand transfer: Integration of the viral DNA template into the target host DNA. This reaction is mediated by tetrameric IN in the nucleus. The IN tetramer is formed when the two DNA-bound IN dimers tetramerize on the viral DNA in presence of the cellular protein LEDGF/p75. IN is in equilibrium between its dimeric and tetrameric states, both of which are required for the various stages of the integration reaction.[10–12]

The IN inhibitors that are currently used in the clinic or under advanced development are strand transfer inhibitors that act by inhibiting the DNA binding of IN.[5] We have recently described the new shiftide approach for the development of IN inhibitors.[13] This approach is based on peptides that shift the oligomerization equilibrium of IN from the active dimer to an inactive tetramer that can barely bind the unprocessed viral DNA and thus is unable to mediate the 3′-end processing step.[12] To act as shiftides, the designed peptides have to bind specifically to the IN tetramer. Since LEDGF/p75 binds specifically to the tetramer of IN, we derived our IN-inhibitory peptides from the IN-binding loops of LEDGF/p75. Indeed, these peptides shifted the IN oligomerization equilibrium toward the inactive tetramer and inhibited IN catalytic activities in vitro as well as HIV-1 replication in infected cells.[13] The shiftide approach bears advantages over the conventional interface dimerization inhibitors, because it overcomes the drawbacks of targeting a protein–protein interaction interface[14] by modulating the oligomerization in a noncompetitive allosteric mechanism.

The HIV-1 Rev protein is a 116-AA viral auxiliary protein that mediates the nuclear export of partially-spliced or unspliced viral RNA.[15,16] We have found that there is an interaction between the HIV-1 IN and Rev proteins.[17] On the basis of this finding, we identified two domains within the HIV-1 Rev protein that mediate its binding to IN. Peptides derived from these binding domains blocked IN enzymatic activity in vitro, and following their penetration into cultured cells, inhibited viral cDNA integration and thus HIV-1 replication.[17] Using fluorescence anisotropy, we have shown that these peptides bind the IN tetramer and as such are potential shiftides. In the current study, we show that the mechanism by which the Rev-derived peptides inhibit IN is a shiftide mechanism: IN was dimeric when bound to the viral DNA, but tetrameric in presence of the peptides. The Rev-derived peptides inhibited the DNA binding of IN as well as its catalytic activity in vitro. The relative order of addition of Rev peptides/LTR DNA had a significant effect on the degree of inhibition, confirming that the inhibition is due to the ability of the peptides to shift the oligomerization equilibrium of the free IN toward a tetramer that has a reduced binding affinity to the viral DNA. We conclude that protein–protein interactions of IN may serve as a general valuable source for shiftide design.

RESULTS

The Rev-Derived Peptides Bind the IN Tetramer

The HIV-1 Rev-derived peptides that inhibit IN activity in vitro and in cells[17] were designed based on a protein–protein interaction of IN,[17] using a similar approach as we have shown in the case of the LEDGF/p75-derived peptides.[13] To establish whether these peptides also inhibit IN in a shiftide mechanism, we tested their effect on the IN oligomeric state using analytical gel filtration (Figure 1a). Unbound IN eluted as a high order oligomer. IN was tetrameric in presence of the Rev-derived peptides, but was dimeric in the presence of LTR DNA, indicating a shift in the oligomerization equilibrium in presence of the peptides. To support the analytical gel filtration results, the IN protein was chemically crosslinked and its profile was then analyzed by SDS-gel electrophoresis. A tetrameric IN appeared only following the incubation of IN with the Rev-derived peptides but not without them (Figure 1b). IN was dimeric following its incubation with the LTR DNA (data not shown). The gel filtration results and the crosslinking study provide a direct evidence for the IN oligomeric state in presence of the peptides and support the fluorescence anisotropy results obtained in our previous studies (see Hill coefficients values in Table I).[17]

The Rev-Derived Peptides Inhibit IN Binding to the Viral LTR DNA

We used fluorescence anisotropy to study whether the shift in the IN oligomerization equilibrium, caused by the Rev-derived peptides, affects the DNA binding of IN. IN bound to a fluorescein-labeled 36-bp double stranded viral LTR DNA with K_d of 34 nM and a Hill coefficient of 2 (Figure 2 and Table I), in agreement with our previous results.[13] This indicates that IN binds the LTR DNA tightly and as a dimer. The Rev-derived peptides significantly inhibited the binding of IN to the viral LTR DNA. The affinity of IN to the DNA was reduced 10-fold from 34 nM without the Rev peptides to 320 nM and 300 nM in presence of Rev 13-23 (1 μM) and Rev 53-67 (1 μM), respectively (Figure 2 and Table I). We conclude that the shiftide activity of the Rev peptides makes them inhibit the DNA binding of IN because they shift IN to a tetrameric state that binds only weakly to the viral DNA.

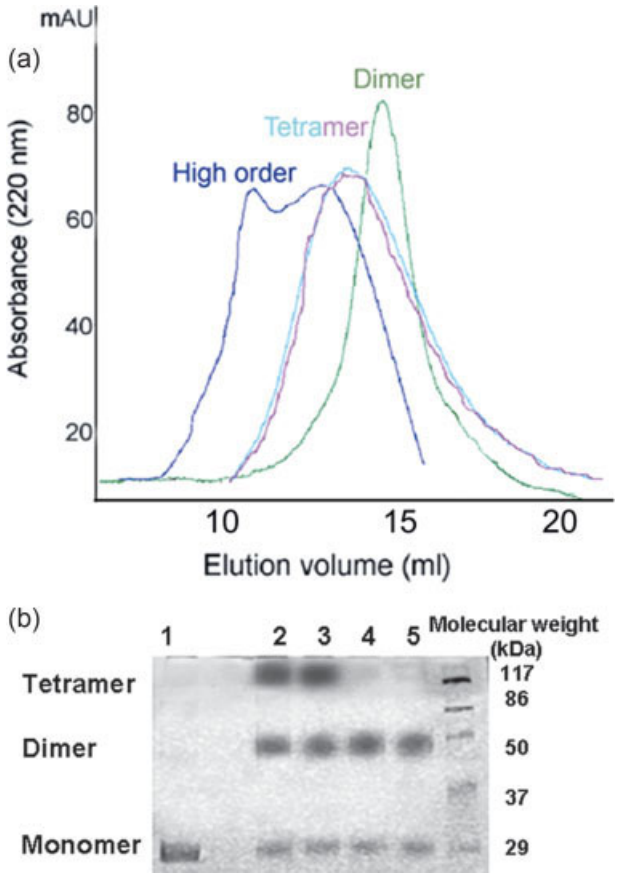

FIGURE 1 The IN-binding Rev-derived peptides are shiftides. (a) The Rev-derived peptides shift the IN oligomerization equilibrium toward the tetramer. Oligomerization of IN in the presence of the peptides was studied using analytical gel filtration. IN 1-288 (14 μM) alone eluted as a high order oligomer (blue). In presence of Rev 13-23 (14 μM, Magenta) or Rev 53-67 (14 μM, Cyan) IN eluted as a tetramer. In presence of 14 μM viral LTR DNA, IN eluted as a dimer (green).(b) The IN protein was incubated with or without the Rev 13-23 or Rev 53-67 peptides and then crosslinked using BS^3 as a crosslinker. The results were analyzed by 10% SDS gel electrophoresis. (1) Noncrosslinked IN-Only a monomeric state can be observed. (2) IN was incubated with Rev 13-23 and was analyzed following crosslinking. As can be seen, the monomeric state disappears and a tetramer appears in addition to the dimer. (3) the same as (2) but in the presence of Rev 53-67. (4-5) Crosslinked IN without peptide. Please note the appearance of monomeric and dimeric states.

Increasing Concentrations of the Rev-Derived Shiftides Inhibit IN Catalytic Activity

To further prove that the Rev peptides inhibit the catalytic activity of IN in a shiftide mechanism, we performed a quantitative in vitro integration assay[8,18]. The addition of increasing concentration of the Rev-derived peptides to a preformed IN-DNA complex blocked the integration reaction. Inhibition became more pronounced when the peptide : IN ratio increased (Figures 3a and 3b). Around 60% inhibition was observed at 5:1 peptide : IN ratio, compared to only around 20% inhibition at 1:1 ratio. The dose response curves indicate that the inhibition by the Rev-derived peptides is specific.

The Order of Addition of the DNA or the Rev-Derived Peptides has a Major Effect on the Extent of Inhibition by the Peptides

We used the quantitative in vitro integration assay[17,18] to determine the effect of the order of addition of the DNA or the Rev-derived shiftides on the IN catalytic activity. When the viral LTR DNA was added to a preformed IN-Rev-derived peptide complex, the IN catalytic activity was inhibited by 70% (Figures 4a and 4b). On the other hand, when the Rev-derived peptides were added to a preformed IN-DNA complex at the same molar ratio 1:1, the IN catalytic activity was only slightly inhibited by 20%. These differences are because when the shiftide is added to free IN, it shifts its oligomerization equilibrium toward a tetramer. This is resulting in a 10-fold reduced binding affinity to the viral DNA, and therefore the inhibition takes place. In the opposite order of addition (DNA first), most IN is already DNA-bound when the peptide is added. The peptide can then shift the equilibrium only of the free IN fraction (see Figure 5). In summary, the order of addition of the various reaction components has an immense effect on the IN catalytic activity, similar to what was described.[19]

DISCUSSION

In the current work, we describe the mechanism of action of IN-inhibitory peptides that were designed based on the interaction of IN with the HIV-1 Rev protein. Our results show that the Rev-derived peptides act as shiftides in a similar mechanism to the LEDGF-derived peptides we previously described. We showed in our previous work that the Rev-derived peptides bind IN and inhibit its activity in vitro and in cells. Here we used gel filtration, crosslinking experiments, fluorescence anisotropy, and quantitative in vitro integration assay to show that the Rev-derived peptides modulate the IN oligomerization equilibrium toward tetramer and what are the consequences of this shift. Our anisotropy results confirm that this tetramer has lower affinity to viral DNA and as a result IN catalytic activity is inhibited. The results of the dose response experiments demonstrate the specificity of the inhibition by the Rev-derived peptides.

The order of addition experiments further support the shiftide mechanism. This mechanism for IN inhibition[13] implies that the relative order of addition of the various com-

Table I Effect of the Rev-Derived Peptides on the Binding of HIV-1 IN to the Viral LTR DNA[a]

(a) IN binding to Rev Peptides[17]			
Peptide	Sequence	K_d Binding to IN (μM)	Hill Coefficient
Rev 13-23	LKTVRLIKFLY	2.8 ± 0.1	3.6 ± 0.5
Rev 53-67	RSISGWILSTYLGRP	6.9 ± 0.1	5.2 ± 0.9
(b) IN Binding to the Viral LTR DNA			
DNA/Peptide	K_d Binding to IN (μM)	Hill Coefficient	
Fl′-LTR DNA only	0.034 ± 0.01	2.0 ± 0.3	
Fl′-LTR DNA + Rev 13-23	0.320 ± 0.02	1.9 ± 0.1	
Fl′-LTR DNA + Rev 53-67	0.300 ± 0.09	2.1 ± 0.2	

[a] Binding studies were carried out using fluorescence anisotropy, as described in the text. The K_d values for peptide binding in section (a) of the table are taken from Ref. 17.

ponents required for integration in vitro will have a profound effect on the biological outcome, as illustrated in Figure 5. In principle, addition of viral DNA to preformed IN-shiftides complex, where the oligomerization state of IN was already shifted toward the tetramer, should result in significant inhibition of IN-DNA binding and as a result inhibition of the IN catalytic activity. This was the case with the Rev-derived peptides as illustrated in the scheme (Figures 5a and 5b). On the other hand, when the shiftide is added to a preformed IN-viral DNA complex, inhibition is weaker because most of the IN is already bound to the DNA and is catalytically active, so that the equilibrium is shifted toward the DNA-bound state. The shiftide can then only bind the IN fraction that is free, shift its oligomerization equilibrium toward the tetramer, and as a result cause inhibition of that fraction of IN. This effect should be dependent on the shiftide:IN molar ratio and inhibition should increase as more shiftide is added. Again, this was the case with the Rev-

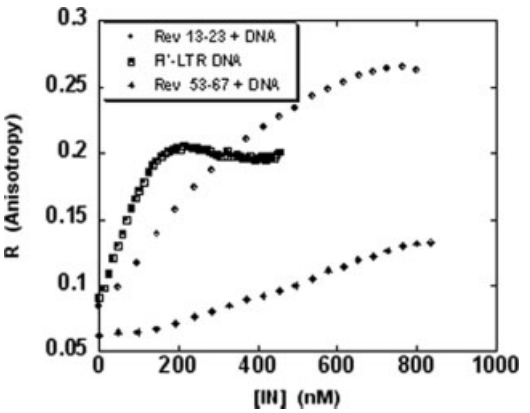

FIGURE 2 The IN-binding Rev-derived peptides inhibit the DNA binding of IN. IN was titrated into fluorescein-labeled HIV-1 LTR DNA (10 nM) alone (■) and in the presence of 1 μM of: Rev 13-23 (●) and Rev 53-67 (◆). Binding affinities and Hill coefficients are shown in Table I(b).

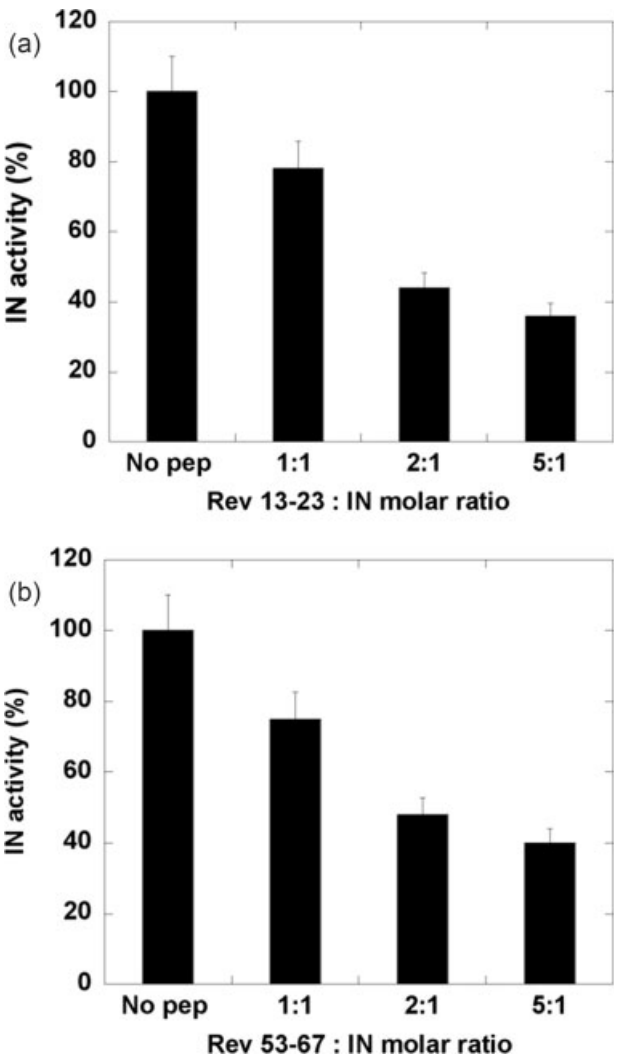

FIGURE 3 The inhibition of the IN catalytic activity is concentration dependent (a and b) Rev-derived peptides (a) Rev 13-23 and (b) Rev 53-67 were added to a preformed IN-DNA complex, when the peptide : IN ratio was increased the inhibition of IN catalytic activity was more pronounced. Each experiment was performed in triplicate and repeated 3 times.

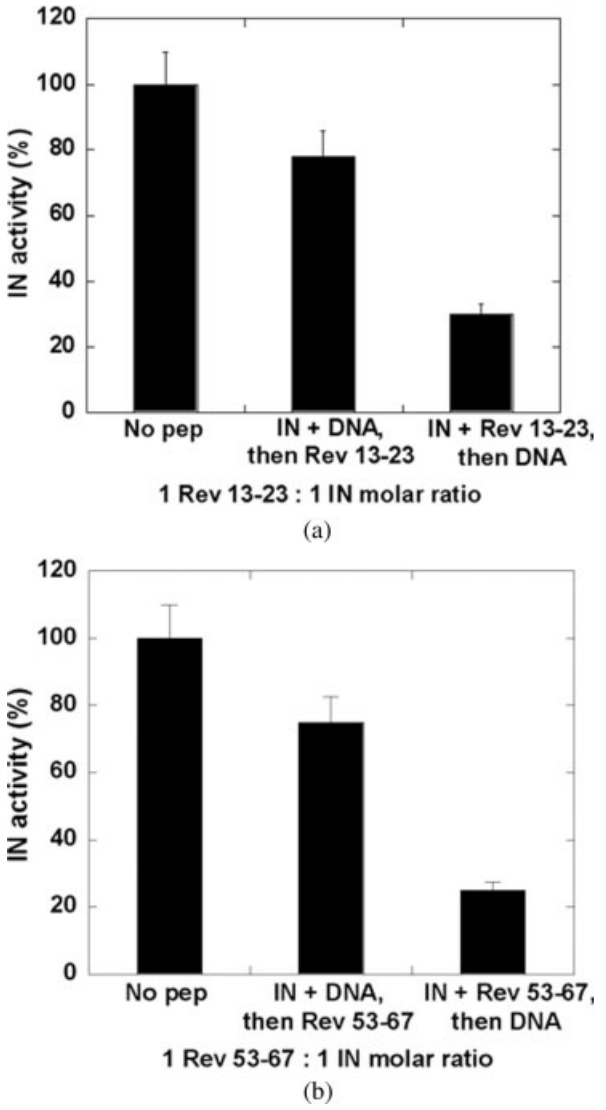

FIGURE 4 The effect of the relative order of addition of the Rev-derived peptides or DNA on IN catalytic activity (a and b). The viral LTR DNA was added to a preformed IN-peptide complex, with (a) Rev 13-23 and (b) Rev 53-67. The IN catalytic activity was then significantly inhibited. However, when Rev-derived peptides were added to a preformed IN-DNA complex, the IN catalytic activity was only slightly inhibited (at 1:1 molar ratio IN:Rev-derived peptides). Each experiment was performed in triplicate and repeated 3 times.

derived peptide (Figure 3a). In summary, the orders of addition experiments of the Rev-derived shiftides or the DNA support the shiftide activity of the Rev-derived peptides.

There are several in vitro assays to determine the IN catalytic activity that are described in the literature. The main differences between these assays are the length of the donor DNA that is used and whether it contains one or two LTR sequences at its termini. Another difference is whether the donor DNA was 3′-processed or unprocessed.[20–26] Previously it was shown that crosslinked tetrameric IN was unable to catalyze one-LTR integration step,[10] confirming the results in the current study. On the other hand, the crosslinked tetrameric IN was able to catalyze the full-site integration step in vitro using a relatively long donor DNA, which was 3′-processed.[10] The crosslinked dimeric IN was involved in the processing and integration of only one viral termini.[10] Crosslinked monomeric IN or IN bound to peptides that block dimerization was unable to catalyze the IN catalytic activity.[20]

In our recent studies as well as in the current work, we describe IN-inhibitory shiftides that were designed based on two protein–protein interactions of IN, with the cellular protein LEDGF/p75 and with the viral protein Rev. Both the Rev-derived peptides and the LEDGF/p75-derived peptides act in a similar mechanism, and they bind IN tetramer and inhibit its DNA binding as well as its catalytic activity in vitro and in vivo.[13,17] Our results imply that protein–protein interaction can be used as a source for the design of shiftides that inhibit the activity of a given oligomeric protein in a noncompetitive mechanism, and not only for the design of competitive inhibitors. We propose that peptides derived from binding interfaces of proteins are a promising starting point for shiftide design.

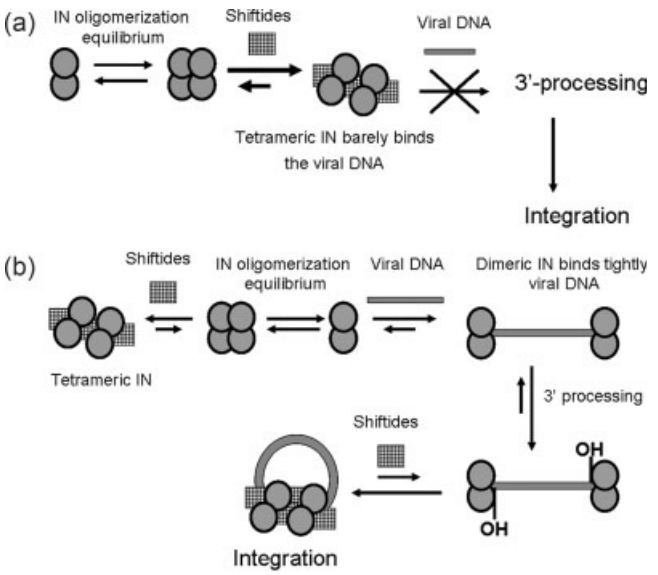

FIGURE 5 The mechanistic basis for the effect of the order of addition on IN catalytic activity in vitro. (a) Forming the IN-shiftide complex before adding the viral DNA results in shifting of the oligomerization state of IN towards a tetramer that hardly binds DNA and consequently in inhibition of IN catalytic activities. (b) Addition of shiftides to a preformed IN-viral DNA complex results in weaker inhibition, which is dependent on the IN:shiftide molar ratio.

We still do not fully understand what in the peptide sequence makes it a shiftide for IN, and is there a general "shifting motif." 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. To elucidate the structural basis for the shiftide activity, it is necessary to reveal the peptide binding sites in the IN protein at its dimeric and tetrameric forms using detailed structural analysis such as NMR or X-ray crystallography. Such studies are currently performed in our laboratory.

MATERIALS AND METHODS

Peptide Synthesis, Labeling, and Purification

Peptides were synthesized on an Applied Biosystems (ABI) 433A peptide synthesizer. The peptides were purified on a Gilson HPLC using a reverse-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 described.[13]

Protein Expression and Purification

The full-length histidine-tagged IN expression vector was a generous gift from Dr. A. Engelman and its expression and purification were performed essentially as described previously.[27]

Analytical Gel Filtration

Analytical gel filtration of IN (10 μM) was performed on an ÄKTA Explorer using a Superose 12 analytical column 30 cm × 1 cm (GE Healthcare-Pharmacia Corp.) equilibrated with buffer: 20 mM Tris pH 7.4, 1M NaCl, and 10% glycerol. Proteins were eluted with a flow rate of 1 ml/min at 4°C and the elution profile monitored by UV absorbance at 220 nm. The column was calibrated with molecular weight standards (GE Healthcare-Pharmacia Corp.).[13]

HIV-1 Integrase Crosslinking Assay

The HIV-1 integrase (1.2 μM final concentration) was incubated with varying amount of peptide at 37°C for 1 h in a pH 7.5 buffer containing 25 mM HEPES, 200 mM NaCl, 1 mM DTT, and 1 mM EDTA. The reaction mixtures were treated with the crosslinking agent BS^3 (25 equiv, Pierce) for 30 min at 37°C. The reaction mixtures were denatured and analyzed by 10% SDS PAGE.[20]

Fluorescence Anisotropy

Measurements were performed at 10°C using a PerkinElmer LS-55 luminescence spectrofluorimeter equipped with a Hamilton Microlab 500 dispenser.[28,29] The fluorescein-labeled LTR DNA sequence was used: 5′-AGACCCTTTTAGTC AGTGTGGAAAATCTCTAG-CAGT-3′, (1 ml, 10 nM in 20 mM Tris buffer pH 7.4, 185 mM NaCl) was placed in a cuvette, and the nonlabeled IN protein (200 μl, ~4 μM), which was preincubated for 30 min with the Rev-derived peptides, was titrated into the fluorescein-labeled LTR DNA (10 nM) in 20 aliquots of 10 μl 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:

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

where R = 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).[13]

Quantitative Estimation of IN Catalytic Activity In Vitro

Determination of the IN enzymatic activity by a quantitative assay was preformed as described.[17,18] Briefly, an oligodinucleotide IN substrate was used, in which one oligo (5′-ACTGCTAGAGATTTTC-CACACTGACTAAAAGGGTC) was labeled with biotin on the 3′ end and the other oligo (5′-GACCCTTTTAGTCAGTGTG-GAAAATCTCTAGCAGT) was labeled with digoxigenin at the 5′ end. When peptide inhibition was studied, the IN (390 nM) was preincubated with the peptide at the indicated molar ratio for 15 min prior to addition of the DNA substrate. In the opposite order of addition, IN was incubated with the DNA substrate and then the peptides were added to the reaction at the indicated molar ratios. Overall IN reaction was followed by an immunosorbent assay on avidin-coated plates as described.[17,18]

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