In Vitro DNA Tethering of HIV-1 Integrase by the Transcriptional Coactivator LEDGF/p75

Melissa McNeely[1]†, Jelle Hendrix[2]†, Katrien Busschots[1], Eline Boons[1], Angélique Deleersnijder[3], Melanie Gerard[3], Frauke Christ[1] and Zeger Debyser[1]*

[1]*Laboratory for Molecular Virology and Gene Therapy, Molecular Medicine, KULeuven and IRC Kulak, Kapucijnenvoer 33, B-3000 Leuven, Flanders, Belgium*

[2]*Photochemistry and Spectroscopy, Division of Molecular and Nanomaterials, KULeuven, Celestijnenlaan 200f, B-3000 Leuven, Flanders, Belgium*

[3]*Laboratory of Biochemistry, IRC, KULeuven-Kortrijk, Etienne Sabbelaan 53, B-8500 Kortrijk, Flanders, Belgium*

Although LEDGF/p75 is believed to act as a cellular cofactor of lentiviral integration by tethering integrase (IN) to chromatin, there is no good *in vitro* model to analyze this functionality. We designed an AlphaScreen assay to study how LEDGF/p75 modulates the interaction of human immunodeficiency virus type 1 IN with DNA. IN bound with similar affinity to DNA mimicking the long terminal repeat or to random DNA. While LEDGF/p75 bound DNA strongly, a mutant of LEDGF/p75 with compromised nuclear localization signal (NLS)/AT hook interacted weakly, and the LEDGF/p75 PWWP domain did not interact, corroborating previous reports on the role of NLS and AT hooks in charge-dependent DNA binding. LEDGF/p75 stimulated IN binding to DNA 10-fold to 30-fold. Stimulation of IN–DNA binding required a direct interaction between IN and the C-terminus of LEDGF/p75. Addition of either the C-terminus of LEDGF/p75 (amino acids 325–530) or LEDGF/p75 mutated in the NLS/AT hooks interfered with IN binding to DNA. Our results are consistent with an *in vitro* model of LEDGF/p75-mediated tethering of IN to DNA. The inhibition of IN–DNA interaction by the LEDGF/p75 C-terminus may provide a novel strategy for the inhibition of HIV IN activity and may explain the potent inhibition of HIV replication observed after the overexpression of C-terminal fragments in cell culture.

Corresponding author. E-mail address:
zeger.debyser@med.kuleuven.be.

† M.M. and J.H. contributed equally to this work.

Abbreviations used: IN, integrase; NLS, nuclear localization signal; LTR, long terminal repeat; PIC, preintegration complex; IBD, integrase binding domain; HIV-1, human immunodeficiency virus type 1; $^1O_2$, singlet oxygen; CR1, charged region 1; MBP, maltose binding protein; FCS, fluorescence correlation spectroscopy; Chaps, 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid; dsDNA, double-stranded DNA.

Introduction

Integration is a critical process in the lentiviral replication cycle and in gene therapeutic applications of lentiviral vectors. After reverse transcription, the viral enzyme integrase (IN) binds to the long terminal repeats (LTRs) of viral cDNA and catalyzes two sequential reactions: 3′-processing and strand transfer.[1,2] During 3′-processing, endonucleolytic cleavage of the CAGT-3′ ends of the viral DNA results in recessed CA-3′-hydroxyl ends. After

this reaction in the cytoplasm of the cell, IN remains associated with the viral cDNA and is transported into the nucleus as part of the preintegration complex (PIC). The PIC associates with the host chromosome, and IN catalyzes the transesterification–ligation reaction in which the reactive 3′-OH groups of the processed viral cDNA are covalently linked to the 5′-phosphate of the host DNA, resulting in a 5-bp single-stranded DNA gap at both viral–host DNA junctions.[3] Once the last two nucleotides from the 5′ ends of the proviral DNA are cleaved off and the gap between the host and the proviral DNA is filled by host DNA repair enzymes, integration is complete.[4] Despite the importance of the process, the exact mechanisms for the IN–DNA/chromatin interaction and integration site selection remain largely unexplained.

Recent studies have shown that the IN-interacting cellular protein LEDGF/p75[5] plays a crucial role in lentiviral replication and integration (for reviews, see Engelman and Cherepanov[6] and Poeschla[7]). LEDGF/p75 is a weak coactivator of general transcription that plays an important role in protection against cellular stress.[8,9] LEDGF/p75 and its splice variant p52 are transcribed from the *psip1* gene; p52 and p75 share homology in their N-terminal domains but have alternative C-terminal domains. In contrast to LEDGF/p75, p52 does not play a role in HIV replication. LEDGF/p75 is a component of the PIC that acts as a chromatin tethering factor, thereby facilitating the interaction between IN and chromatin.[10] After initial identification and validation of the interaction between LEDGF/p75 and IN,[5,11] the crucial role of LEDGF/p75 in HIV replication has been corroborated through different approaches, including mutagenesis, RNA interference, transdominant inhibition, and knockout.[12–18]

LEDGF/p75 contains several DNA/chromatin binding motifs in its N-terminal domain, as well as an integrase binding domain (IBD) in the C-terminal domain (residues 347–429)[19–23] (Fig. 1a). The predominant N-terminal putative DNA/chromatin binding motif is the PWWP domain, which has been postulated to be involved in DNA binding due to its similarity to the PWWP domain of the hepatoma-derived growth factor.[24] Although the PWWP domain of LEDGF/p75 poorly binds DNA *in vitro*,[19,25] the domain clearly localizes to chromatin in cells and binds chromatinized template DNA *in vitro*.[10,25] The nuclear localization signal (NLS) and AT-hook-like motifs downstream of the PWWP domain are also described as candidates for DNA binding (Fig. 1a). These motifs[10] affect neither the chromatin binding function of LEDGF/p75 nor the function of LEDGF/p75 as a chromatin tether for IN.[26] *In vitro* studies indicate a role for these elements in DNA binding.[25] Through C-terminal interaction with IN and N-terminal binding to

chromatin, LEDGF/p75 effectively tethers the viral IN to the host cell chromatin, thereby facilitating integration.

Lentiviruses preferentially integrate into transcriptionally active regions of the chromatin.[3] Recently, it has become clear that LEDGF/p75 is the determinant for this lentiviral integration site selection.[18,26–29] After LEDGF/p75 depletion, integration site preference resembles that of murine leukemia virus more, with a higher frequency of integration close to transcription start sites. Although the N-terminal domain of LEDGF/p75 is thought to direct the viral integration into transcriptionally active sites, no sequence-specific DNA motif has been identified.

At the time of its discovery, recombinant LEDGF/p75, but not p52, was shown to stimulate IN activity using mini-HIV DNA substrates.[5] Addition of recombinant LEDGF/p75 increases the solubility of recombinant IN.[30] According to Yu *et al.*, the stimulation of IN activity by LEDGF/p75 requires addition of LEDGF/p75 to IN before the formation of the IN–donor DNA complex, suggesting an interaction of IN and LEDGF/p75 prior to reverse transcription.[31] In a study by Raghavendra and Engelman, LEDGF/p75 was shown to stimulate half-site integration, whereas full-site concerted integration was inhibited.[32] The authors speculated that LEDGF/p75 might interfere with full-site integration by interfering with IN oligomerization. Since no inhibition of the catalytic activity of preassembled IN complexes was observed, LEDGF/p75 was postulated to play a role in the viral life cycle after the formation of the PIC. Pandey *et al.*, however, demonstrated activation of concerted integration by LEDGF/p75, but only when low molar ratios of LEDGF/p75 and IN were used.[33] Concerted integration was again inhibited when the molar ratio of LEDGF/p75 to IN exceeded 1. In their hands, the IBD itself could stimulate or inhibit the concerted integration, albeit at a lower efficiency. According to Cherepanov *et al.*, the IBD alone is unable to stimulate IN strand transfer activity but competitively counteracts the stimulatory effects of the full-length protein.[34] Finally, as evidenced by Botbol *et al.*, human immunodeficiency virus type 1 (HIV-1) integration into a chromatinized template is also stimulated by LEDGF/p75.[25] Furthermore, LEDGF/p75 has been shown to stabilize IN subunit–subunit interaction and to promote IN tetramerization.[35,36] Also, IN tetramers, not dimers, have been observed in association with viral DNA ends using atomic force microscopy.[37]

Here we have extended the biochemical studies to the modulation of the interaction of HIV-1 IN with DNA by LEDGF/p75. We established a novel assay based on Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen) technology to
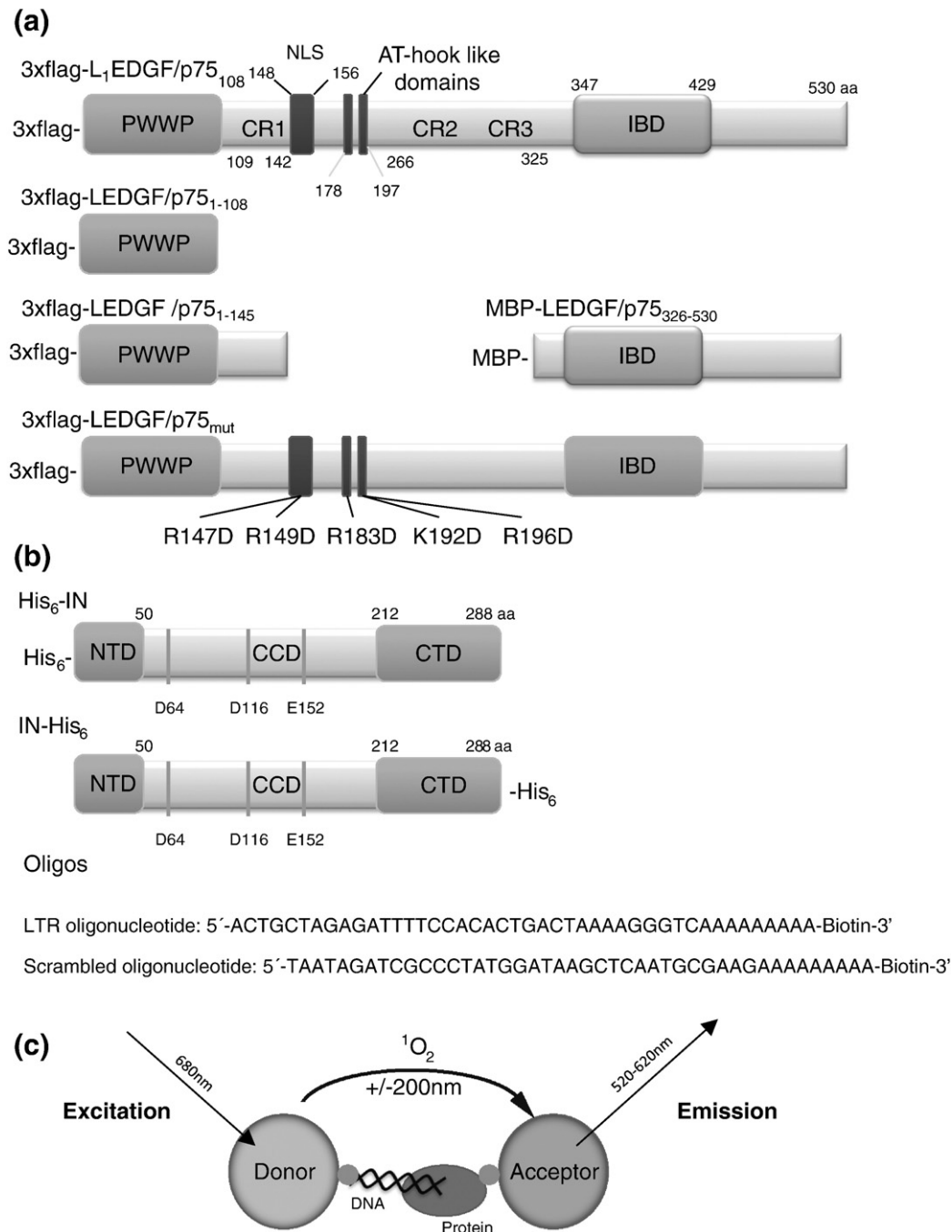
Fig. 1. Constructs used in AlphaScreen binding assays. (a) Domain structure of LEDGF/p75 and schematic representation of 3×FLAG-tagged constructs. LEDGF/p75 contains a C-terminal IBD and a combination of DNA and chromatin binding elements in the N-terminus. CR1–3 = charged regions 1–3; NLS = nuclear localization signal; MBP = maltose binding protein. (b) Domain structure and schematic representation of HIV IN, double-stranded oligonucleotides, and oligonucleotide LTR mimicking the lentiviral LTR, a known DNA substrate for IN, as well as a scrambled version of this oligo. NTD = N-terminal domain; CCD = catalytic core domain; CTD = C-terminal domain. (c) AlphaScreen technology measures biological interactions and is based on the use of donor and acceptor beads that are coated with functional groups for bioconjugation. The direct interaction of two molecules will lead to the transfer of $^1O_2$ after excitation, resulting in the emission of light.

measure the interaction of recombinant HIV-1 IN and LEDGF/p75 with oligonucleotide DNA. In this assay, LEDGF/p75 stimulated IN binding to DNA 10-fold to 30-fold. We thus provide an *in vitro* model of LEDGF/p75 mediating tethering of IN to DNA.

Results

HIV-1 IN binds with low affinity to DNA

We used AlphaScreen to study the *in vitro* DNA binding of IN. Both IN and DNA were engineered to contain an affinity label: recombinant IN carrying an N-terminal or a C-terminal His_6-tag (Fig. 1b) and

DNA, i.e. double-stranded oligonucleotides mimicking the 35-bp 3′ end of the U5-LTR of HIV cDNA present after reverse transcription, carrying a biotin affinity label and an extra poly(A) linker to avoid steric hindrance (Fig. 1b). In the assay, these affinity labels interact strongly with ~250-nm beads: streptavidin-functionalized 'donor' beads and Ni^{2+}-chelate-functionalized 'acceptor' beads. When IN and DNA interact, donor and acceptor beads come
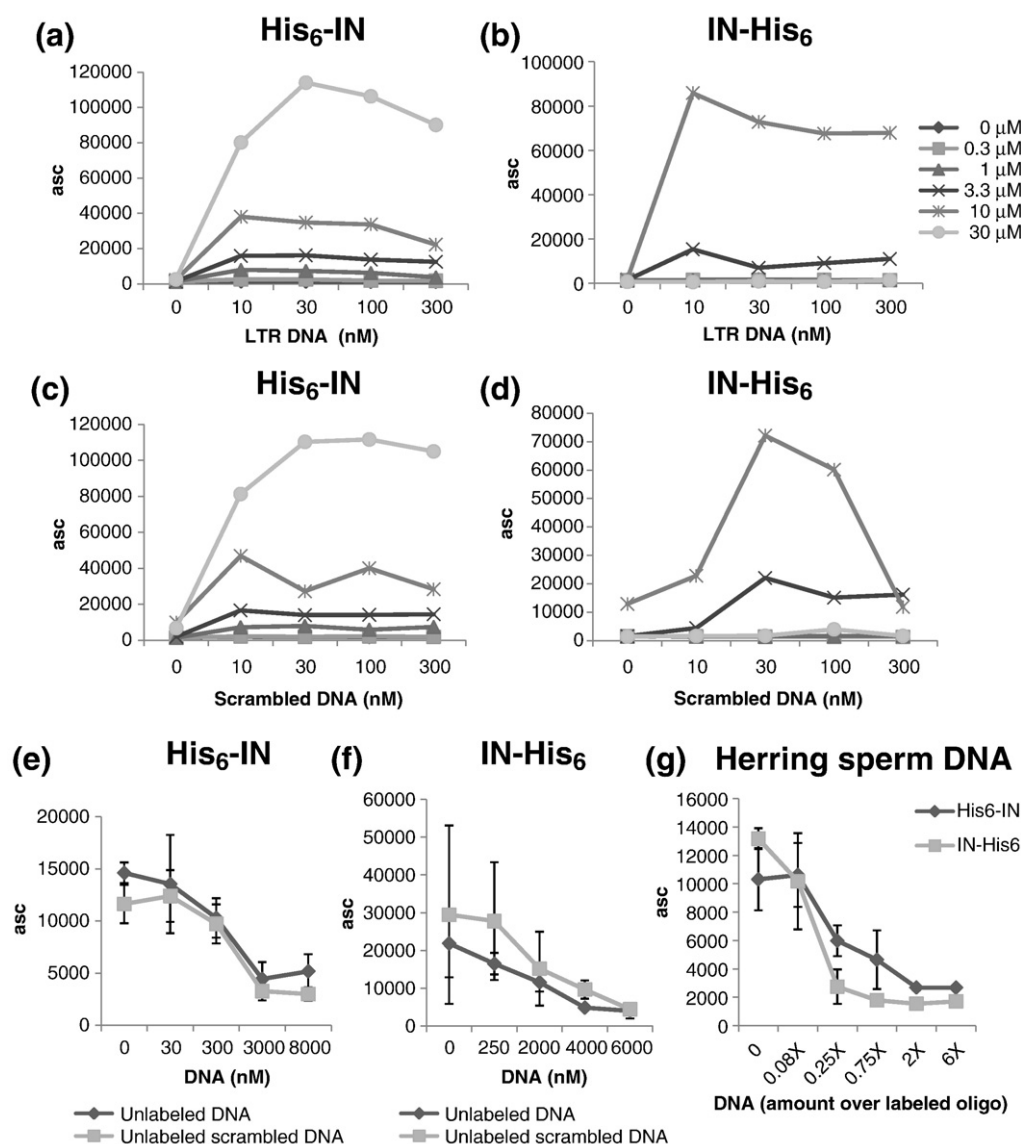

Fig. 2. DNA binding properties of HIV IN. Cross-titrations show optimal concentration ranges for the binding of HIV IN to LTR and scrambled oligonucleotides. (a) His_6–IN and (b) IN–His_6 are titrated against LTR and (c and d) scrambled oligonucleotide. IN concentrations between 0 and 30 μM in 1:3 serial dilutions were used. Concentrations were determined by Bradford analysis after the removal of aggregates from the preparation. Data points for cross-titrations are in singlet. asc = AlphaScreen luminescence counts. (e) His_6–IN (6 μM) and 30 nM biotin-labeled oligo were outcompeted by unlabeled oligo and randomized oligo. (f) IN–His_6 (6 μM) and 15 nM biotin-labeled oligo were outcompeted by unlabeled oligo and randomized oligo. (g) Addition of unlabeled unspecific DNA in the form of sheared herring sperm DNA easily abolished the binding signal between IN and DNA. Averages and standard deviations of a triplicate experiment are shown.

in close proximity. Irradiation of the solution with 680-nm light triggers singlet oxygen ($^1O_2$) production and release from the donor bead. A luminescence fluorescence reaction in the acceptor bead catalyzed by $^1O_2$ causes emission of 520–620 nm light, which can finally be registered (Fig. 1c).

We first cross-titrated His_6–IN or IN–His_6 with DNA to determine the optimal concentrations for measuring the interaction (Fig. 2), as earlier work demonstrated that the position of the affinity label may affect the binding of IN to LEDGF/p75.[30] We readily saw the binding of His_6–IN to 10–300 nM DNA (Fig. 2a). Increasing His_6–IN concentrations between 0 and 30 μM resulted in a higher AlphaScreen signal. At DNA concentrations above 30 nM, we observed the 'hooking effect,' a known effect in AlphaScreen assays: whereas all DNA molecules can interact with acceptor beads at low DNA concentrations, at DNA concentration exceeding the binding capacity of the beads, non-bead-bound DNA molecules compete with bead-bound DNA for interaction with IN, effectively lowering the AlphaScreen signal. For IN–His_6, the signal reached a maximum between 10 and 30 nM DNA (Fig. 2b). We also measured binding to scrambled DNA, in which the previously used LTR sequence was scrambled but still carried the poly(A) linker and the biotin label (Fig. 1b). The results for the scrambled DNA were in the same range (Fig. 2c and d) as for the LTR DNA, suggesting that IN does not bind DNA specifically. For subsequent experiments, we used LTR DNA substrate at a concentration of 15 nM.

Next, we titrated 15 nM DNA with His_6–IN or IN–His_6. Both sigmoidal binding curves (data not shown) were fitted to Eq. (11). For His_6–IN, $K_d = 3.8 \pm 0.3$ μM; for IN–His_6, $K_d = 3.5 \pm 0.7$ μM (Table 1). To ensure that the increase in signal was not due to aggregation of IN, we also assayed the signal at high concentrations of IN (up to 8 μM) and in the absence of LTR, but this resulted in only background counts (data not shown). Furthermore, increasing concentrations of untagged DNA (Fig. 2e and f), untagged scrambled DNA (Fig. 2e and f), and sheared herring sperm DNA (Fig. 2g) could abolish the signal, corroborating that the signal was indeed due to the IN–DNA interaction. This indicates again

Table 1. Relative K_d values for His_6–IN and IN–His_6

Protein	K_d (μM)[a]	R^2
His_6–IN	3.8 ± 0.3	0.9483
IN–His_6	3.5 ± 0.7	0.9643

[a] K_d was determined by fitting to Eq. (11) the binding curve of recombinant IN and dsDNA LTR oligonucleotide, as measured with the AlphaScreen assay. Values are expressed as average ± SD for three independent measurements. R^2 quantifies the goodness of fit for nonlinear regression. An R^2 value of 1 represents a perfect fit.

that IN does not bind to DNA in a sequence-specific manner. In summary, under the experimental conditions of our AlphaScreen assay, IN was found to interact nonspecifically with DNA with low affinity ($K_d = 3$–4 μM).

High-affinity binding of LEDGF/p75 to DNA is mediated by NLS and AT hooks

Next, we measured the interaction of LEDGF/p75 and DNA in the AlphaScreen assay by using 3×FLAG-labeled LEDGF/p75, donor beads coated with anti-FLAG antibody, and the same LTR oligonucleotide as for the experiments on IN. LEDGF/p75, at concentrations ranging from 3 to 300 nM, interacted readily with DNA, at concentrations ranging from 1 to 10 nM (Fig. 3a). The hooking effect was observed at DNA concentrations above 3 nM for all tested LEDGF/p75 concentrations and at LEDGF/p75 concentrations of about 100–300 nM. Next, we titrated 3 nM DNA with increasing concentrations of 3×FLAG-LEDGF/p75 (Fig. 3b). The AlphaScreen signal increased hyperbolically; after fitting to Eq. (7), a K_d of 13.1 ± 2.1 nM was determined (Table 2).

Truncated versions of LEDGF/p75, namely 3×FLAG-LEDGF/p75$_{1-108}$ containing the PWWP domain only and 3×FLAG-LEDGF/p75$_{1-145}$ containing the PWWP domain and the charged region 1 (CR1) domain (Fig. 1a), did not bind at all to DNA in our assay (data not shown). We next generated a mutated form of LEDGF/p75, called 3×FLAG-LEDGF/p75^{mut}, in which we reversed the charge of five putatively critically important amino acids in the NLS and AT-hook-like domains (R147D, R149D, R183D, K192D, and R195D) and assayed DNA binding. In cross-titrations, binding was seen only at the highest concentrations of DNA (Fig. 3c). After the titration of 300 nM DNA with 3×FLAG-LEDGF/p75^{mut}, fitting of the resulting binding curve with Eq. (7) gave a K_d of 380.5 ± 101.5 nM, which represents a 29-fold reduction in affinity for DNA with respect to wild-type 3×FLAG-LEDGF/p75 (Fig. 3d, Table 2). Taken together, these results indicate that LEDGF/p75 binds DNA with high affinity under the experimental conditions of our AlphaScreen assay ($K_d = 13.1$ nM), the PWWP and PWWP–CR1 domains do not appear to bind DNA, and the NLS and AT hook elements constitute important contributions to this affinity.

LEDGF/p75 drastically enhances the binding of HIV-1 IN to DNA

Next, we investigated the impact of LEDGF/p75 on the IN–DNA interaction. We first performed detailed cross-titrations by adding increasing amounts of untagged recombinant LEDGF/p75 to various concentrations of IN in the presence of
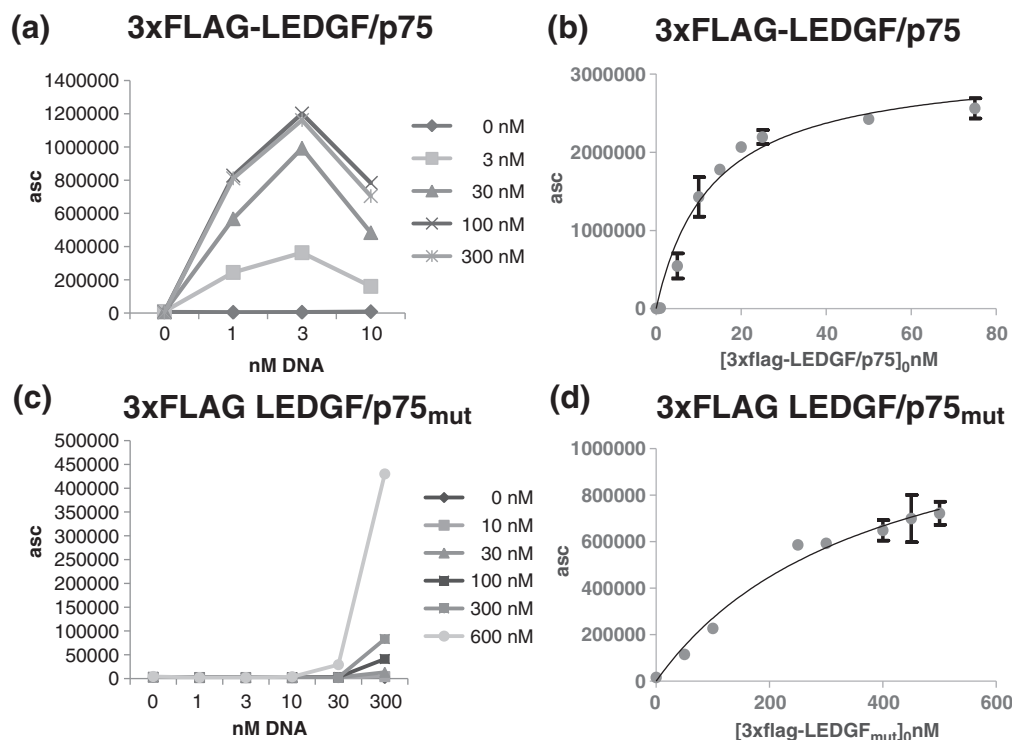
Fig. 3. Binding of LEDGF/p75 to DNA. (a) Cross-titrations of 3×FLAG-LEDGF/p75 with DNA shows the optimal binding range in the low nanomolar range for both 3×FLAG-LEDGF/p75 and oligonucleotide. Data points are in singlet for a representative experiment. (b) K_d determination of 3×FLAG-LEDGF/p75 for DNA. 3×FLAG-LEDGF/p75 is titrated between 0 and 75 nM in the presence of 3 nM DNA to determine the relative K_d. (c) Cross-titration of 3×FLAG-LEDGF$_{mut}$ (from 0 to 500 nM) with 0–300 nM DNA shows a reduced affinity for DNA. (d) Relative K_d for 3×FLAG-LEDGF$_{mut}$ was determined in the presence of 300 nM DNA. Averages and standard deviations of a duplicate experiment are shown for relative K_d. The continuous line is a fit to Eq. (7).

15 nM LTR oligonucleotide. Addition of LEDGF/p75 increased the observed affinity of both His$_6$–IN and IN–His$_6$ for DNA (Fig. 4a and b). For His$_6$–IN, the effect was only observed clearly at 100 nM LEDGF/p75, but the effect was most striking with

IN–His$_6$, where LEDGF/p75 drastically increased DNA binding at all concentrations, even at low concentrations of IN where no baseline DNA binding was measurable in the absence of LEDGF/p75. The order of addition had no significant effect on signal intensity (data not shown). We again determined an apparent K_d for the binding of IN to 15 nM DNA in the presence of 100 nM LEDGF/p75 by fitting the binding curve with Eq. (11) (Fig. 4c and d, Table 3). For His$_6$–IN, $K_d = 0.42 \pm 0.01$ μM; for IN–His$_6$, $K_d = 0.11 \pm 0.0$ μM. Addition of LEDGF/p75 thus increased the apparent affinity of IN–DNA by 10-fold (His$_6$–IN) to 32-fold (IN–His$_6$).

The stimulatory effect of LEDGF/p75 on IN–DNA interactions was also confirmed in an enzyme-linked immunosorbent assay (ELISA)-based IN enzymatic activity assay (Fig. 5a). LEDGF/p75 increased the enzymatic activity of IN by 50% (Fig. 5a, first and second columns). Preincubation of IN with the DNA before addition of LEDGF/p75 increased the activity of IN by 200% (Fig. 5a, third column). Preincubation of the DNA with LEDGF/p75 before addition of IN also increased the activity of IN, but not to a higher extent as preincubation

Table 2. Interaction of wild-type and mutant LEDGF/p75 with DNA

Protein	K_d (nM)[a]	R^2
3×FLAG-LEDGF/p75	13.1 ± 2.1	0.9704
3×FLAG-LEDGF/p75^{mut}	380.5 ± 101.5	0.9704
3×FLAG-LEDGF/p75$_{1–108}$	No binding	
3×FLAG-LEDGF/p75$_{1–145}$	No binding	

[a] K_d was determined with the AlphaScreen assay for wild-type or mutant LEDGF/p75 binding to dsDNA LTR oligo. 3×FLAG-LEDGF/p75$_{1–108}$ contains the PWWP domain only, and 3×FLAG-LEDGF/p75$_{1–145}$ contains the PWWP and CR1 domains. In 3×FLAG-LEDGF/p75^{mut}, the charge of five putatively critically important amino acids in the NLS and AT-hook-like domains is reversed (R147D/R149D/R183D/K192D/R195D). Results are given as mean ± SD for two independent measurements. R^2 quantifies the goodness of fit for nonlinear regression. An R^2 value of 1 represents a perfect fit.
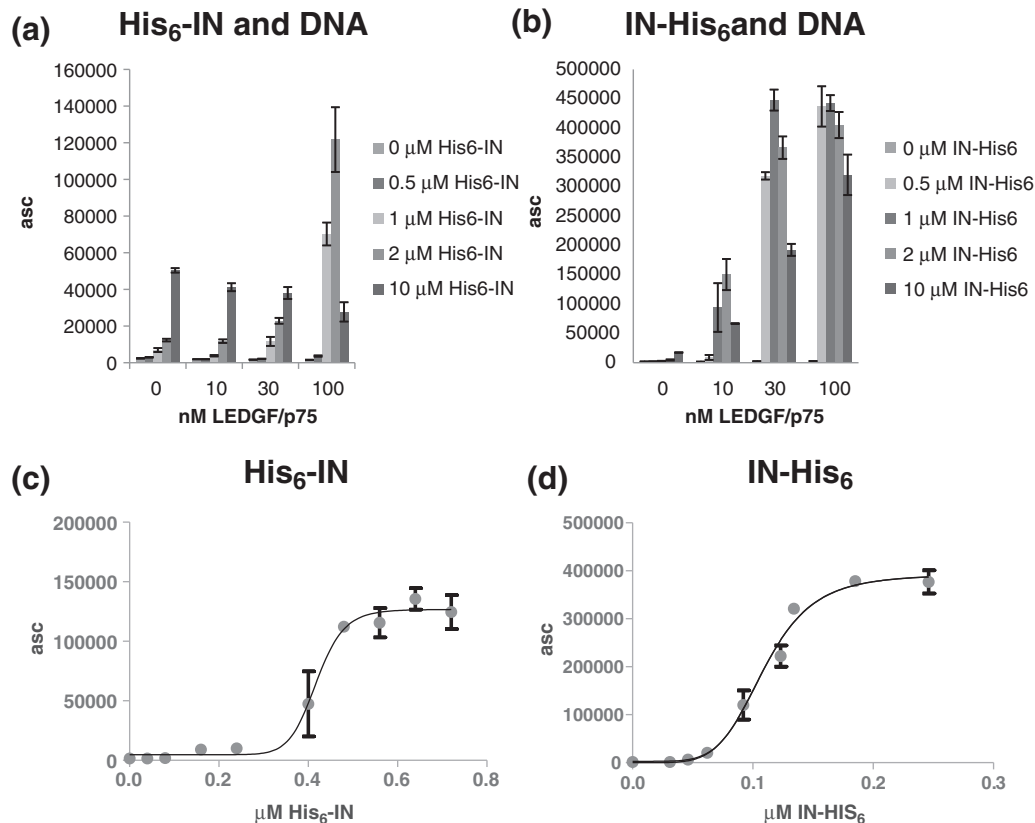
Fig. 4. Effect of LEDGF/p75 on IN–DNA binding. (a) His$_6$–IN (0–10 μM) and (b) IN–His$_6$ (0–10 μM) were incubated with 15 nM DNA, and increasing amounts of LEDGF/p75 (0–100 nM) were added to each IN and DNA combination. Data are derived from a triplicate experiment, with averages and standard deviation shown. (c) Relative K_d determination of His$_6$–IN for 15 nM DNA in the presence of 100 nM LEDGF/p75. The continuous line is a fit to Eq. (11). (d) Relative K_d determination of IN–His$_6$ for 15 nM DNA in the presence of 100 nM LEDGF/p75. K_d determination data points are in triplicate, with averages and standard deviation of data points shown. The continuous line is a fit to Eq. (11).

with both IN and DNA. In summary, an increase in IN–DNA affinity by LEDGF/p75 can be translated into an increase in the activity of IN.

Solubility of HIV-1 IN in the presence of oligonucleotide DNA or LEDGF/p75

We set up a solubility assay to exclude that addition of DNA or LEDGF/p75 induced IN

Table 3. LEDGF/p75 increases the interaction of His$_6$–IN or IN–His$_6$ with DNA

Protein	Additional protein	K_d (μM)[a]	R^2
His$_6$–IN	LEDGF/p75 (100 nM)	0.42 ± 0.01	0.9920
IN–His$_6$	LEDGF/p75 (100 nM)	0.11 ± 0.00	0.9810

[a] K_d was determined with the AlphaScreen assay for recombinant IN, with a His$_6$-tag at the N-terminus (His$_6$–IN) or the C-terminus (IN–His$_6$) binding to dsDNA LTR oligo in the presence of 100 nM LEDGF/p75. Results are given as mean±SD for two independent measurements. R^2 quantifies the goodness of fit for nonlinear regression. An R^2 value of 1 represents a perfect fit.

aggregation, confounding the readout in AlphaScreen. Addition of DNA did not affect the solubility of either IN (Fig. 6a; Supplementary Fig. 2), indicating that the increase in AlphaScreen signal upon addition of DNA is due to the IN–DNA interaction and not due to DNA-induced IN aggregation. Addition of LEDGF/p75 increased the solubility of both His$_6$–IN and IN–His$_6$ (Fig. 6b; Supplementary Fig. 2), as previously reported.[30] His$_6$–IN proved less soluble than IN–His$_6$. The addition of LEDGF/p75 increased the soluble fraction of IN–His$_6$ even at the lowest salt concentration. Both variants of IN contain soluble and insoluble fractions at 150 mM NaCl. Finally, we added maltose binding protein (MBP) LEDGF/p75$_{326–530}$ (Fig. 1a) to IN in the solubility assay (Fig. 6c; Supplementary Fig. 2). This C-terminal fragment of LEDGF/p75 lacks the known DNA binding motifs and therefore does not interact with the DNA substrate but retains IN binding affinity. This fragment also increased the solubility of IN (Fig. 6c; Supplementary Fig. 2).
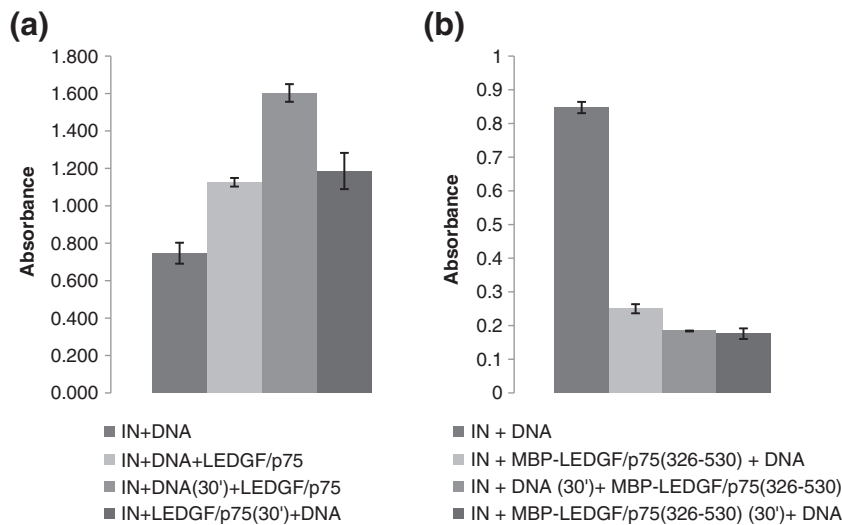
(a)

(b)

Fig. 5. Overall enzymatic activity of IN as measured with ELISA. (a) Order of addition assays shows the overall stimulation of His$_6$–IN activity with addition of LEDGF/p75. Stimulation was greatest when 450 nM His$_6$–IN was prebound to 70 nM oligonucleotide for 30 min in reaction buffer at 4 °C, followed by addition of 200 nM LEDGF/p75. (b) Order of addition assays shows the overall inhibition of activity with addition of MBP-LEDGF/p75$_{326–530}$, with inhibition being greatest at 200 nM IN incubated with 70 nM oligonucleotide and 100 nM MBP-LEDGF/p75$_{326–530}$. The extent of inhibition was not affected by the order of addition. Averages and standard deviations of a triplicate experiment are shown.

Role of LEDGF/p75 domains in the modulation of IN–DNA interaction

In order to unravel the mechanism by which LEDGF/p75 increases the affinity of IN for DNA, we tested three different LEDGF/p75 mutants. First, a truncated LEDGF/p75 lacking all DNA binding elements but retaining the IBD (MBP-LEDGF/p75$_{326–530}$) was tested. We also assayed 3×FLAG-LEDGF/p75^{mut} (R147D, R149D, R183D, K192D, and R195D), 3×FLAG-LEDGF/p75^{D366A} (a previously described full-length protein with a mutation in the IBD that renders the protein unable to interact with IN *in vitro*),[34] and p52 (a splice variant of LEDGF/p75 with an identical N-terminal IBD but lacking the C-terminal IBD).

First, we determined the binding of His$_6$–IN and IN–His$_6$ to DNA (15 nM) in the presence of increasing concentrations of MBP-LEDGF/p75$_{326–530}$ (Fig. 7). No significant stimulation of the signal was apparent at lower concentrations of MBP-LEDGF/p75$_{326–530}$ (Fig. 7a and b), in contrast to the significant stimulation apparent with full-length LEDGF/p75. In fact, addition of MBP-LEDGF/p75$_{326–530}$ resulted in a concentration-dependent inhibition of the IN–DNA interaction. In a separate experiment, we determined the MBP-LEDGF/p75$_{326–530}$

concentration required to inhibit the DNA binding of 8 μM His$_6$–IN and 6 μM IN–His$_6$ by 50% (IC$_{50}$) (Fig. 8a and b, Table 4). For both His$_6$–IN and IN–His$_6$, the IC$_{50}$ was 0.04 μM. MBP alone did not affect IN–DNA binding (data not shown). As described above, 3×FLAG-LEDGF/p75^{mut} shows severely decreased DNA binding in the AlphaScreen assay but retains the IN binding properties of LEDGF/p75,[38,39] as well as an intact PWWP domain. The mutant strongly inhibited IN–DNA binding (His$_6$–IN IC$_{50}$ = 0.08 μM; IN–His$_6$ IC$_{50}$ = 0.65 μM) (Fig. 8c and d, Table 4). Addition of 3×FLAG-LEDGF/p75^{D366A} that carries all functional DNA binding domains—but a mutation that renders the IBD unable to interact with IN— inhibited IN–DNA binding to a lesser extent, with IC$_{50}$ values of 1.79 μM (His$_6$–IN) and 2.26 μM (IN–His$_6$) (Fig. 8e and f, Table 4). p52, the splice variant of LEDGF/p75 with an identical N-terminus but no IBD, inhibited poorly, with IC$_{50}$ values of 3.65 μM (His$_6$–IN) and 9.39 μM (IN–His$_6$) (Fig. 8g and h, Table 4). To investigate whether the inhibitory effect of the C-terminal fragment could be extrapolated to an inhibition of the enzymatic activity of IN, we performed the ELISA-based activity assay. Inhibition of the enzymatic reaction was seen when 100 nM MBP-LEDGF/p75$_{326–530}$ was added to the

Fig. 6. Binding partners and solubility of HIV IN. Addition of increasing amounts of NaCl increased IN solubility. HIV IN (1.5 μM) was incubated with a binding partner in the presence of a gradient from 100 to 500 mM NaCl. By comparison of the relative amount of IN present in the soluble fraction *versus* the insoluble fraction at varying salt concentrations, one can visualize the effect of an additional component on IN solubility. (a) IN solubility in the presence of DNA. Addition of 3 μM oligonucleotide did not affect IN solubility *in vitro*. (b) IN solubility in the presence of LEDGF/p75. LEDGF/p75 displayed a positive effect on recombinant IN solubility, with the fraction of insoluble IN decreasing in the presence of LEDGF/p75 and with the fraction of soluble IN increasing in the presence of LEDGF/p75. (c) IN solubility in the presence of MBP-LEDGF/p75$_{326–530}$. Addition of MBP-LEDGF/p75$_{326–530}$ also increased the solubility of recombinant IN.

(a) His6-IN   -oligo   +oligo

(b) His6-IN   -LEDGF/p75   +LEDGF/p75

(c) His6-IN   -MBP-LEDGF/p75₃₂₆₋₅₃₀   +MBP-LEDGF/p75₃₂₆₋₅₃₀
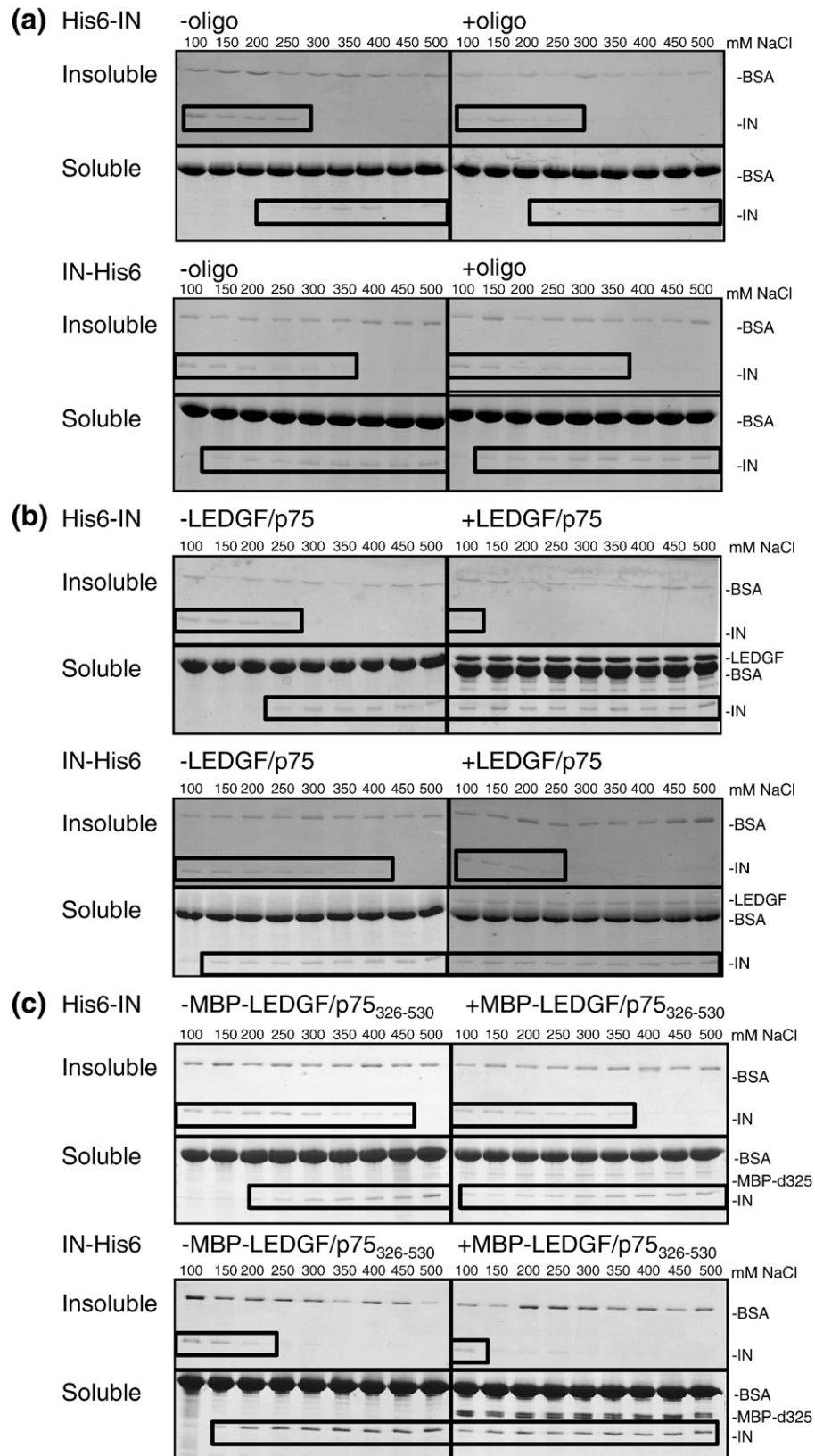
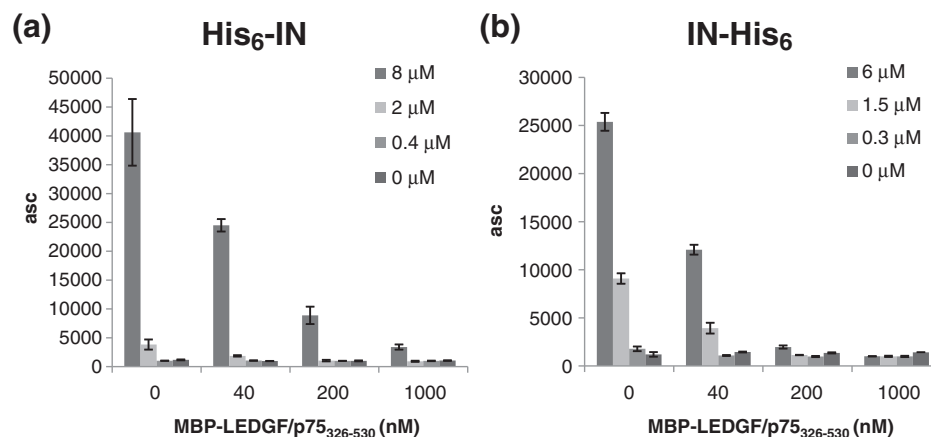**Fig. 6** (*legend on previous page*)

Fig. 7. Addition of MBP-LEDGF/p75$_{326-530}$ to the IN–DNA binding assay. (a) His$_6$–IN (0–8 μM) was incubated with 15 nM DNA in the presence of 0–100 nM MBP-LEDGF/p75$_{326-530}$. (b) IN–His$_6$ (0–6 μM) was incubated with 15 nM DNA in the presence of 0–100 nM MBP-LEDGF/p75$_{326-530}$. MBP-LEDGF/p75$_{326-530}$ did not stimulate IN–DNA binding at lower concentrations of IN and showed an inhibitory effect on IN–DNA binding at higher concentrations of IN. Data are derived from a triplicate experiment, with averages and standard deviations shown. asc = AlphaScreen luminescence counts.

assay that contained 70 nM oligo and 200 nM IN (Fig. 5b). The inhibition was observed irrespective of the order of addition of the different partners.

Discussion

In a series of experiments, we have utilized a novel *in vitro* IN–DNA binding assay based on the AlphaScreen technology to study the effect of LEDGF/p75 and LEDGF/p75 variants on the IN–DNA interaction. Using IN with His$_6$-tags at either terminal, we have looked at the binding of IN to a double-stranded oligonucleotide mimicking the viral LTR, as well as to a scrambled oligonucleotide. We have used our AlphaScreen assay to estimate the apparent K_d values of both IN and LEDGF/75 for DNA, as well as the apparent K_d value for IN binding to DNA in the presence of LEDGF/p75. Finally, we have determined which domains of LEDGF/p75 are responsible for increased *in vitro* binding of IN to DNA.

Quantifying IN–DNA interaction with AlphaScreen

DNA binding of IN has been studied extensively in the past with electrophoretic mobility shift assays,[40,41] Southwestern blot analysis,[42,43] nitrocellulose filter assays,[44–47] chemical or UV cross-linking of IN to DNA,[48–51] surface plasmon resonance,[52,53] fluorescence correlation spectroscopy,[54] and fluorescence anisotropy.[55–57] Although these assays were generally used to study the protein domain structure and sequence specificity of binding, binding affinities have also been calculated. Literature K_d values for IN binding to LTR oligonucleotides vary considerably from 0.3 to 700 nM under different assay conditions.[30,53,54,57–59]

An important buffer component that has been shown to have a large influence on the apparent K_d is NaCl.[58] In our assays, protein stock solutions were diluted in buffer containing 150 mM NaCl for binding experiments. In buffers with NaCl concentrations ranging from 0.05 to 0.2 M, the affinity of IN for DNA ranged from a K_d of 0.1 μM to a K_d of 2 μM.[58] We obtained similar low micromolar apparent K_d values for His$_6$–IN–DNA (K_d=3.8±0.3 μM) and IN–His$_6$–DNA (K_d=3.5±0.7 μM) (Table 1). Fluorescence correlation spectroscopy (FCS) analysis yielded a K_d of 200 or 700 nM in the presence of 50 or 130 mM NaCl, respectively.[54] With surface plasmon resonance, performed with IN carrying solubility mutations (C56S, C65S, and C280S), a K_d of 20 nM was calculated.[59] Fluorescence anisotropy measurements

Fig. 8. Inhibition of IN–DNA binding by the addition of various LEDGF/p75 truncations and mutants. (a) His$_6$–IN (8 μM) was incubated with 15 nM DNA, followed by the addition of 0–0.5 μM MBP-LEDGF/p75$_{326-530}$, to determine IC$_{50}$ by log-inhibitor-*versus*-response analysis. (b) IN–His$_6$ (6 μM) was incubated with 15 nM DNA, followed by addition of 0–0.5 μM MBP-LEDGF/p75$_{326-530}$, to determine IC$_{50}$. The following IC$_{50}$ log-inhibitor-*versus*-response curves were performed in the same fashion. (c) The effect of 0–0.5 μM 3×FLAG-LEDGF/p75^{mut} on His$_6$–IN and DNA binding, as well as on IN–His$_6$ and DNA binding (d). (e) The effect of 0–0.6 μM p52 on His$_6$–IN and DNA binding, as well as on IN–His$_6$ and DNA binding (f). (g) The effect of 0–0.5 μM 3×FLAG-LEDGF^{D366A} on His$_6$–IN and DNA binding, as well as on IN–His$_6$ and DNA binding (h). Data are derived from a triplicate experiment, with averages and standard deviations shown. asc = AlphaScreen luminescence counts.

resulted in a K_d of 65 nM in the presence of 50 mM salt, but much lower affinities were obtained in the presence of 300 mM.[57] We also show that IN binds to both specific and scrambled double-stranded oligonucleotides with low affinity (Fig. 2), in line with previous observations.[58]
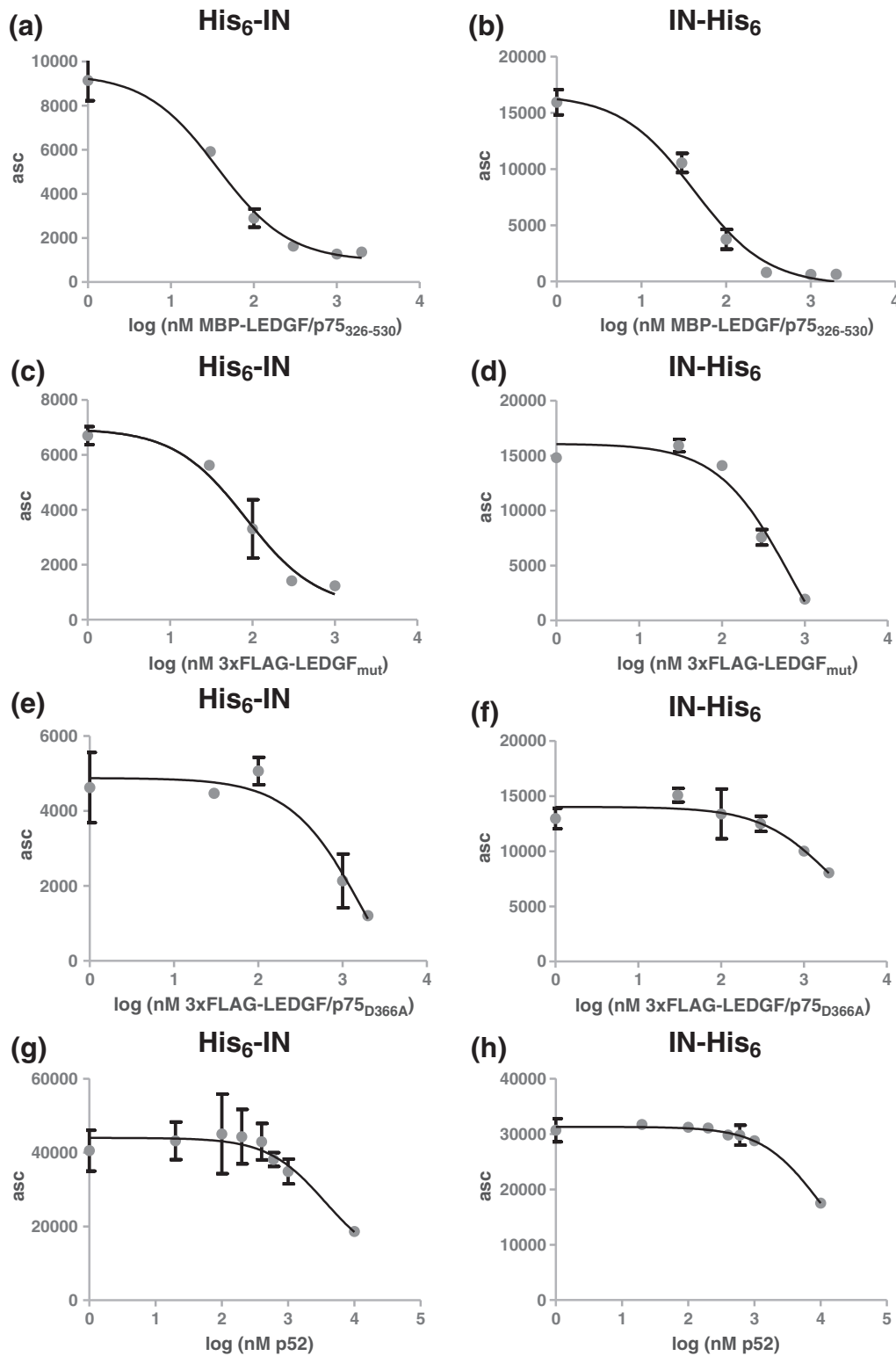

Fig. 8 (*legend on previous page*)

Table 4. Inhibition of IN–DNA interaction by LEDGF/p75 mutants

Protein	Inhibitory protein	IC$_{50}$ (μM)[a]	R^2
His$_6$–IN	MBP-LEDGF/p75$_{326-530}$	0.04 ± 0.001	0.9552
IN–His$_6$	MBP-LEDGF/p75$_{326-530}$	0.04 ± 0.001	0.9755
His$_6$–IN	3×FLAG-LEDGF/p75^{mut}	0.08 ± 0.002	0.8882
IN–His$_6$	3×FLAG-LEDGF/p75^{mut}	0.65 ± 0.001	0.9513
His$_6$–IN	3×FLAG-LEDGF/p75^{D366A}	1.79 ± 0.004	0.8200
IN–His$_6$	3×FLAG-LEDGF/p75^{D366A}	2.26 ± 0.006	0.6385
His$_6$–IN	p52	3.65 ± 0.002	0.7065
IN–His$_6$	p52	9.39 ± 0.002	0.9457

[a] Interaction of 8 μM His$_6$–IN or 6 μM IN–His$_6$ with 15 nM dsDNA LTR oligo was determined by AlphaScreen in the presence of increasing concentrations of MBP-Δ325, 3×FLAG-LEDGF^{mut}, 3×FLAG-LEDGF^{D366A}, or p52. The IC$_{50}$ values were determined using a log-inhibitor-*versus*-response analysis of the data curves and represent the protein concentration that inhibits the interaction by 50%. Results are given as mean ± SD for three independent measurements. R^2 quantifies the goodness of fit for nonlinear regression. An R^2 value of 1 represents a perfect fit.

Absolute affinities are based on the IN monomer concentration. Since IN is known to form higher-order complexes, their presence can affect correct concentration determination. In our previous FCS analysis, nonspecific aggregate-bound DNA may have resulted in an overestimation of DNA binding affinity.[54] In this study, we have therefore removed IN precipitates before AlphaScreen analysis and have chosen to include 7.5 mM of the detergent 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (Chaps) in our assay buffer because it has been shown that detergents help to avoid nonspecific protein aggregation.[60] The inclusion of Chaps in our assay buffer might also explain in part why our determined apparent K_d for IN–DNA is slightly higher than what is generally reported in the literature.

Of note, we observed a certain degree of cooperative binding of IN to DNA (data not shown; Fig. 4). Hill coefficients of around 2 have been observed before when fitting IN–DNA binding curves, suggesting that DNA induced IN dimerization or tetramerization.[57,61] Higher-order stoichiometries of IN have been shown to be induced by DNA binding[54] and have even been shown to be important for the catalytic activity of IN.[56,62] However, care is advised when interpreting these AlphaScreen results; the high degree of cooperativity observed here can be due to the nature of the AlphaScreen assay beads. Multiple independent IN–DNA contacts might lead to a synergistic increase in the overall binding affinity when donor and acceptor beads are added—a phenomenon commonly referred to as avidity. Unlike many other IN–DNA binding assays, the AlphaScreen assay thus is very sensitive to stoichiometric changes in the IN–DNA complex: an increased apparent affinity between the reporter

beads might be observed upon dimerization/oligomerization of IN, again due to an avidity effect rather than an affinity effect. This might explain why we observed apparent cooperativity higher than reported.[57,61]

In summary, determinants such as ionic strength, detergents, and spinning may explain the rather low affinities for IN binding to DNA under our reaction conditions. Also, the inert nature of insoluble recombinant IN in the AlphaScreen assay to measure DNA binding could also lead to an overestimation of IN concentration. Relative values in the absence or in the presence of LEDGF/p75 are, however, highly consistent between FCS and AlphaScreen studies and are likely to be relevant.

LEDGF/p75 interacts strongly with oligonucleotide DNA and increases the apparent DNA binding affinity of HIV-1 IN

In contrast to our previous FCS assay for DNA binding, the novel AlphaScreen-based assay no longer requires proteins to polymerize upon binding to DNA. This assay is thus ideally suited for studying the effect of LEDGF/p75 on the IN–DNA interaction. First, we determined the affinity of LEDGF/p75 for DNA. The K_d determined (K_d = 13.1 nM) is more correct than our previously published value of K_d = 1110 nM[30] and is ~300-fold lower than the affinity of the IN–DNA interaction (K_d ~ 4.3 μM), in line with reports that LEDGF/p75 interacts with DNA/chromatin *in vivo* with a much higher affinity than IN.[63–65] Untagged LEDGF/p75 stimulates IN–DNA binding 10-fold with His$_6$–IN (K_d = 0.42 μM). Stimulation of IN–His$_6$–DNA binding is 32-fold (K_d = 0.11 μM). These experiments are in line with our previously documented pull-down experiments where we demonstrated that the affinity of LEDGF/p75 for IN–His$_6$ is higher than its affinity for His$_6$–IN,[30] which has been suggested to be due to a direct interaction of the N-terminus of IN with LEDGF/p75.[63,64] The N-terminus of IN is also known to be a requirement for high-affinity binding to LEDGF/p75, with the positively charged IBD interacting with negatively charged residues in the N-terminus.[11,66,67] How do these findings relate to our previous analysis using FCS?[30] There, we measured IN–DNA binding by analyzing fluorescent spikes in solution, which reflected high-molecular-weight IN–DNA complexes (aggregates). The K_d for His$_6$–IN bound to a 21-mer double-stranded LTR oligonucleotide was estimated at 719 ± 52 nM, which decreased to 31.6 ± 3.9 nM in the presence of 166 nM LEDGF/p75,[30] a 23-fold decrease, well in line with the results obtained with our new assay. Similar results were obtained for the binding of IN to an unspecific DNA oligo, with a K_d value of 323 ± 12.5 nM for His$_6$–IN and a

K_d value of 50 ± 11 nM for His$_6$–IN in the presence of LEDGF/p75.[30]

The clear sigmoid shape of the IN–LEDGF/p75–DNA binding curve (Fig. 4c and d) can be interpreted in terms of a LEDGF/p75-stimulated increase in the DNA and IN content per complex. LEDGF/p75 has a high affinity for DNA, and it has been shown that IN is present at least as a dimer in solution.[35,68] Since a dimer of IN can already interact with two LEDGF proteins, more than one oligo is likely tethered to the same IN complex via LEDGF/p75. This results in a LEDGF/p75-concentration-dependent increase in the number of contacts between DNA and the donor bead and between IN and the acceptor beads, and thus an apparent increase in the affinity, before beads are saturated with IN.

Measuring protein–DNA interaction without the interference of protein aggregation or altered protein solubility

In the presence of LEDGF/p75, IN–DNA binding is apparently increased 10-fold to 30-fold, and the underlying mechanism is of great interest. Our AlphaScreen approach was strictly controlled for the signal produced by protein aggregation. If addition of oligo were to induce IN aggregation leading to signal, then the binding curves would not accurately reflect DNA binding to IN. Buffers were optimized to maximize the solubility of recombinant protein by use of a Hepes buffer containing Chaps detergent[58] at 150 mM NaCl, and preexisting aggregates were removed from IN preparations before addition of the binding partners. In our solubility assay (Fig. 6), we could show that addition of LTR oligo did not induce aggregation under assay conditions. The AlphaScreen signal measured in the absence of oligo and at high IN concentrations remained at background levels both with and without LEDGF/p75. Additionally, adding unlabeled oligo to our outcompetition assays did not result in an increase in signal, which would be the case if oligos induced aggregates and, as a consequence, produced false binding signal (Fig. 2). Addition of unlabeled DNA resulted in a steep decrease in AlphaScreen counts, clearly indicating that the signal is due to protein–DNA interactions between labeled IN and labeled DNA. To exclude LEDGF/p75-mediated aggregation of IN, we tested two LEDGF/p75 mutants. Addition of a DNA-binding-deficient mutant LEDGF/p75 or the MBP-LEDGF/p75$_{326-530}$ deletion mutant, both of which retain interaction with IN,[34] resulted in a decrease—instead of an increase—in binding signal.

Previously, we have shown that the solubility of IN increases in the presence of LEDGF/p75.[30] Here, we confirm that finding in solubility assays (Fig. 7) and furthermore demonstrate that the position of

the His$_6$-tag (N-terminal *versus* C-terminal) also influences the solubility of IN, with the N-terminally tagged His$_6$–IN shown to be less soluble than the C-terminally tagged protein. Here we additionally show that MBP-LEDGF/p75$_{326-530}$ also increases the solubility of IN. Since titration with this truncated version of LEDGF/p75 did not increase the binding signal of IN for DNA (Fig. 7), the increased signal upon addition of LEDGF/p75 cannot be a result of the increased solubility of IN. Strikingly, the addition of MBP-LEDGF/p75$_{326-530}$ to our binding assay had an inhibitory effect at higher concentrations.

Inhibition of IN–DNA binding by nonfunctional LEDGF/p75 tethers

Surprisingly, upon addition of IN-interacting LEDGF/p75 mutants to our IN–DNA binding assay, both MBP-LEDGF/p75$_{326-530}$ (i.e., IBD) and 3×FLAG-LEDGF/p75^{mut} strongly inhibited the binding of IN to DNA. The less pronounced reduction in IN–DNA binding after addition of p52 or 3×FLAG-LEDGF/p75^{D366A} (Fig. 8e–h, Table 4) likely reflects the background competition for DNA binding.

The inhibition of IN–DNA interaction by IBD is certainly in line with previous studies on the effect of the IBD on HIV integration. One study found the IBD unable to stimulate IN strand transfer activity *in vitro*, competitively counteracting the stimulatory effects of LEDGF/p75.[34] Likewise, the C-terminal fragment was unable to stimulate integration into chromatinized templates.[25] Inhibition of concerted integration by IBD has been documented.[35] McKee *et al.* postulated that LEDGF/p75, but also the IBD by itself, could stabilize the quaternary structure of HIV IN, affecting the enzymatic activity because of restricted protein flexibility.[35] Pandey *et al.* demonstrated a 2-fold to 3-fold activation of concerted integration by IBD at low concentrations.[33] At higher concentrations of IBD, inhibition of concerted integration was again observed. This phenomenon was also seen for full-length LEDGF/p75. The potent inhibition of HIV-1 replication by IBD overexpression of the IBD in cell culture[17,38] has been ascribed to an outcompetition of functional LEDGF/p75 by these C-terminal fragments lacking chromatin binding capacities. Our LEDGF/p75$_{326-530}$ fragment, apart from the affinity tag, is identical with the one used in cell culture (Figs. 7 and 8a and b). We have also shown a strong inhibitory effect using full-length LEDGF/p75 with nonfunctional NLS and AT-hook-like domains. In our hands, these domains mediate double-stranded DNA (dsDNA) binding *in vitro*, confirming previous studies.[25,69] Addition of the 3×FLAG-LEDGF/p75^{mut} to the IN–DNA binding assay strongly inhibited IN–DNA interaction.

Mechanism of the LEDGF/p75-mediated stimulation of IN–DNA interaction

The observed increase in apparent affinity between IN and DNA upon addition of LEDGF/p75 can be explained through two possible mechanisms: (i) LEDGF/p75 might allosterically alter the intrinsic affinity of IN for DNA, or (ii) LEDGF/p75 might indirectly tether IN to DNA. It has been reported that LEDGF/p75 binding to IN stabilizes a quaternary structure compatible with a tetramer or an octamer of IN.[35] Although the latter finding was derived in the absence of DNA, LEDGF/p75 may also increase IN–DNA interaction through an allosteric effect involving the overall conformation of the IN multimer (Fig. 9c). Earlier reports have shown that addition of LEDGF/p75 to IN enzymatic activity tests increases the activity of IN.[70] Still, increased activity may only be secondary to a higher affinity of the IN–LEDGF/p75 complex for DNA. If the first mechanism is correct, and LEDGF/p75 allosterically alters the affinity of IN for DNA, addition of a deletion mutant of LEDGF/p75 (MBP-LEDGF/p75$_{326–530}$) harboring only the IBD would have the same effect as full-length LEDGF/p75. We clearly showed that this is not the case (Fig. 7). We also tested whether 3×FLAG-LEDGF/p75^{mut}, containing intact PWWP domain and IBD but lacking functional NLS and AT hooks, could stimulate IN–DNA. This mutant showed severely decreased DNA binding in the AlphaScreen with a K_d of 380 nM, a 30-fold reduction in binding over wild-type LEDGF/p75 (Fig. 8c and d, Table 4). More importantly, this mutant also showed a strong inhibitory effect on IN–DNA binding. Finally, with the measured K_d values of IN–DNA (3.8 and 3.5 μM) and LEDGF/p75–DNA (13.1 nM), and with the knowledge that the IN–LEDGF interaction is a high-affinity interaction ($K_d \sim 10.9$ nM),[70] we have simulated the steady-state binding equilibrium of their ternary complex under conditions similar to the AlphaScreen assay (Supplementary Fig. 1). If the AlphaScreen signal is proportional to both IN–DNA and IN–LEDGF/p75–DNA, DNA tethering of LEDGF/p75 by IN likely contributes more to the observed signal than IN–DNA interaction. In conclusion, LEDGF/p75 does not stimulate the intrinsic affinity of IN for DNA, and our observed increase in apparent IN–DNA affinity can most likely be explained simply through indirect LEDGF/p75-mediated DNA tethering of IN.

How does LEDGF/p75 tether IN to DNA? The PWWP domain on its own does not bind DNA in our assay (Table 2); 3×FLAG-LEDGF/p75 bound the double-stranded LTR oligo with a K_d of 13.1 nM, but a truncated form of LEDGF/p75 (3×FLAG-LEDGF/p75$_{1–108}$) consisting of only the PWWP domain of LEDGF/p75 displayed no *in vitro* DNA binding. This leaves the NLS and AT-hook-like domains as possible mediators of naked DNA binding. Indeed, when crucial charged amino acids (R147D, R149D, R183D, K192D, and R195D)
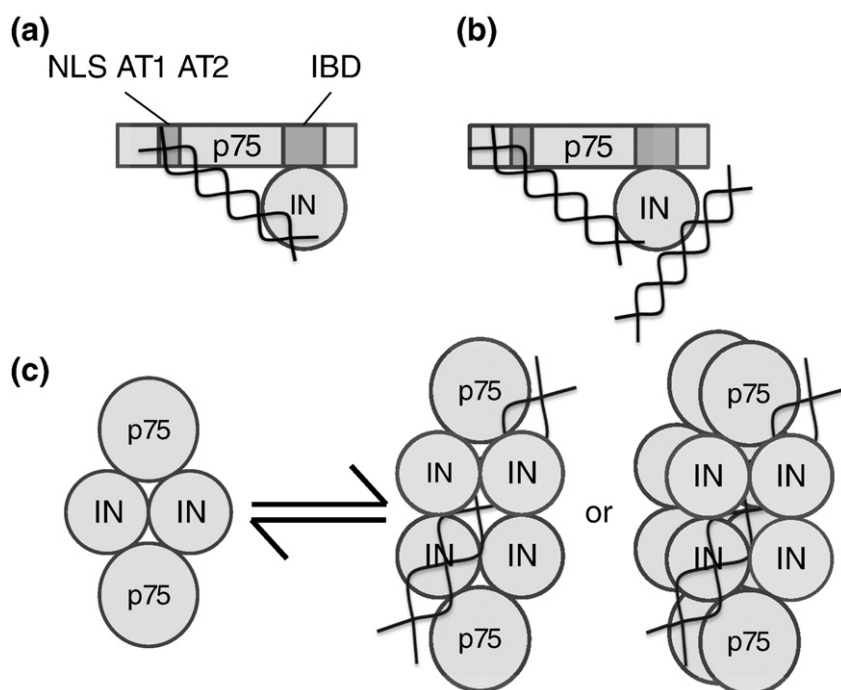

Fig. 9. Putative models for the underlying mechanism of the LEDGF/p75-induced stimulation of IN–DNA binding. (a) LEDGF/p75 binds DNA via the NLS and AT-hook-like motifs in its N-terminal domain. Concurrently, the IBD in the C-terminal domain binds IN, thereby facilitating IN binding to DNA. This model mimics the proposed *in vivo* tethering role of LEDGF/p75. (b) In the second proposed model, IN and LEDGF/p75 each interact with different DNA substrates, which are then brought together through IBD-mediated LEDGF–IN binding. (c) It is also plausible that LEDGF/p75 stabilizes a quaternary structure by shifting the equilibrium in favor of a IN tetramer–LEDGF/p75 complex (or even an IN octamer–LEDGF/p75 complex) compared with an IN dimer–LEDGF/p75 complex. LEDGF/p75 could thus increase IN–DNA interaction through an allosteric effect involving the overall conformation of the IN multimer. Since addition of IN-interacting LEDGF/p75 mutants does not have a stimulatory effect, this model is less likely.

were changed to inverse the charge, DNA binding was severely inhibited, consistent with previous findings.[69] The signal in the cross-titrations was low, and 3×FLAG-LEDGF/p75[mut] had a K_d of 380.9 nM. Since the PWWP domain has been implicated as important for chromatin binding of the PIC during integration into the cell,[10,25,69] we can deduce that such interaction is not mediated by direct DNA binding. Thus, from our findings, we can deduce that LEDGF/p75 binds to IN via the IBD, and the NLS and AT-hook-like domains of LEDGF/p75 bind the LTR oligo, tightening the complex of IN and DNA.[28] This model reflects the proposed *in vivo* function of LEDGF/p75 as a molecular tether that links the viral PIC to the cellular chromatin (Fig. 9). Unfortunately, due to the nature of our assay, we cannot distinguish if IN and LEDGF/p75 bind to the same DNA substrates or to two different DNA substrates (Fig. 9b). It is possible that the latter holds true and that both oligonucleotides are brought together through the IN–IBD interaction. Both models could account for the increase in signal due to the addition of LEDGF/p75.

How does this work relate to HIV infection of the cell? Chromatinized template DNA has been used as an experimental stand-in for genomic DNA in *in vitro* experiments.[25] HIV integration into the polynucleosome template was 12 times more efficient than when naked DNA was used, and the PWWP domain of LEDGF/p75 reportedly played a predominant role in binding to chromatinized template. Although the effect of histone-induced DNA conformation cannot be excluded, the potent stimulation of IN–DNA binding by LEDGF/p75 in our assay was measured in the absence of histones. *In vivo*, the observed affinity of IN for DNA/chromatin is rather low in the absence of endogenous LEDGF/p75.[63–65] In the presence of LEDGF/p75, IN forms a stable complex with LEDGF/p75 that has a strongly reduced nuclear mobility (Maertens *et al.*;[71] J.H., personal communication). Dynamic chromatin scanning of LEDGF/p75 is also decelerated ∼80-fold after coexpression of IN (J.H., personal communication), revealing an even stronger *in vivo* tethering of IN than described here *in vitro*. Likely, the *in vivo* situation represents a combined effect of AT-hook-mediated and NLS-mediated DNA tethering and PWWP-domain-mediated chromatin tethering.

Conclusion

In summary, we have developed an *in vitro* IN–DNA binding assay and have demonstrated that LEDGF/p75 has a stimulatory effect on IN–DNA binding *in vitro*. This assay recapitulates the tethering function of LEDGF/p75, since the interaction between IBD and IN, on one hand, and the interaction between LEDGF/p75 and DNA, on the other hand, were absolutely required. The PWWP domain of LEDGF/p75 is not required for the interaction with naked DNA. Two nonfunctional LEDGF/p75 tethers that cannot interact with DNA inhibit IN–DNA interaction. Understanding of this inhibition may lead to new therapeutic approaches.

Materials and Methods

Cloning of mutants

To generate 3×FLAG-LEDGF/p75$_{1–108}$, 3×FLAG-LEDGF/p75$_{1–145}$, and 3×FLAG-LEDGF/p75[mut], we followed the SLIM mutagenesis protocol[72] using the following primer sets to modify the pCPNatFlag plasmid construct:[73]

for 3×FLAG-LEDGF/p75$_{1–108}$: F1-5′-CCGGCTGCTAA-CAAAGCCCG-3′, F2-5′-TAGAATTCGAAGCTT-GATCCGGCTGCTAACAAAGCCCG-3′, R1-5′-AACATCAGATGATGCATTTGATTGTTTAG-3′, and R2-5′-ATCAAGCTTCGAATTCTAACATCAGATGATG-CATTTGATTGTTTAG-3′;

for 3×FLAG-LEDGF/p75$_{1–145}$: F1 and F2 the same as described above, R1-5′-TTTTGGAGTAGTTATGT-CAACTGCTTTAG-3′, and R2-5′-ATCAAGCTTC-GAATTCTATTTTGGAGTAGTTATGTCAACTGCTT-TAG-3′.

To generate 3×FLAG-LEDGF/p75[mut]: for the mutation of the NLS, R147D R149D: F1-5′-GCAGAAAAACAAG-TAGAAACTGAGGAG-3′, F2-5′-AGGGGGGATAAG-GATAAG GCAGAAAAACAAGTAGAAACTGAGGAG, R1-5′-TCTGGCAGCTTTTGGAGTAGTTATGTC-3′, and R2-5′-CTTATCCTTATCCCCCCTTCTGGCAGCTTTTG-GAGTAGTTATGTC-3′.

To generate the AT hook 2 mutations (K192D R196D): F1-5′-CAGATCCTCGAGGCGATCCCAAAATGG-TAAAACAGCCCTGTCC-3′, F2-5′-CAAAATGG-TAAAACAGCCCTGTCC-3′, R1-5′-GGATCGCCTC-GAGGATCTGGAATCTTGACTTCTGTAGCTG-3′, and R2-5′-GAATCTTGACTTCTGTAGCTG-3′.

To generate the AT hook 1 mutation (R183D) with the primer set 5′-GTGAGTCCTAAAAGAGGGGATCCTG-CAGCTACAGAAG-3′ and Δ325 R primer 5′-CGTCGTACGGGTACTGGCCGGCCTGG, the Kirsch–Joly method[74] was used.

Oligonucleotides

Two different DNA oligonucleotides resembling either the LTR sequences or a scrambled control were used: LTR oligo, 5′-ACTGCTAGAGATTTTCCACACT-GACTAAAAGGGTCAAAAAAAAA-biotin-3′ (Sigma-Aldrich, Bornem, Belgium). To generate the scrambled oligo, we randomized the same sequence: 5′-TAATA-GATCGCCCTATGGATAAGCTCAATGCGAA-GAAAAAAAAA-biotin-3′.

Both oligos were labeled with biotin at the 3′ end on the sense strand and then annealed to their corresponding unlabeled antisense strand (LTR oligo complementary strand, 5′-TTTTTTTTTGACCCTTTTAGTCAGTGTG-GAAAATCTCTAGCAGT-3; for scrambled oligo, 5′-TTTTTTTTTCTTCGCATTGAGCTTATCCATAGGGC-GATCTATTA-3′) (Fig. 1b). Identical oligonucleotides lacking the biotin label were used in competition experiments. Sheared herring sperm DNA (Sigma-Aldrich) was also used in competition experiments in which the binding signal was competed out by unlabeled LTR oligo, unlabeled scrambled oligo, or herring sperm DNA in order to demonstrate sequence specificity of binding.

Purification of recombinant proteins

HIV-1 IN was expressed from pRP1012[52] (His₆–IN) or pKB-IN6H[64] (IN–His₆) and purified as previously described.[64] The stock solution of His₆–IN and IN–His₆ contained 30 mM (Tris pH 7.4), 800 mM NaCl, 7.5 mM Chaps, 5 mM DTT, and 10% (vol/vol) glycerol. Nontagged LEDGF/p75 and p52 were expressed from pCP-Nat plasmids and purified as described previously.[64] The stock solution of LEDGF/p75 contained 30 mM Tris (pH 7.0), 150 mM NaCl, 5 mM DTT, and 10% (vol/vol) glycerol. Tagged 3×FLAG-LEDGF/p75 and mutants thereof were produced from pCPNatFlag plasmids,[73] as described for nontagged LEDGF/p75. MBP-Δ325 was expressed and purified as described previously.[38] The stock solution of MBP-LEDGF/p75₃₂₆₋₅₃₀ contained 50 mM Tris (pH 7.2), 150 mM NaCl, and 50% (vol/vol) glycerol.

AlphaScreen assay

AlphaScreen (Fig. 1c) was performed according to the manufacturer's protocol (PerkinElmer, Zaventem, Belgium). The standard reaction buffer consisted of 50 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 7.5 mM Chaps, 0.01% (vol/vol) Tween-20, and 0.1% (wt/vol) bovine serum albumin, or (for LEDGF/p75 K_d determination) 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 0.01% (vol/vol) Tween-20, and 0.1% (wt/vol) bovine serum albumin. All working solutions were diluted in buffers at 150 mM salt. Because the solubility of IN decreased upon storage, we separated large aggregates from soluble IN by centrifugation and determined the absolute protein concentration prior to each experiment with the Bradford assay (Bio-Rad Laboratories, Nazareth Eke, Belgium). Proteins were diluted in the reaction buffer in a total volume of 25 μl in 384-well Optiwell™ microtiter plates (PerkinElmer) and incubated for 60 min at 4 °C. When testing multiple binding partners, we first added double-stranded oligonucleotides, followed by IN and LEDGF/p75 or mutant LEDGF/p75. After the initial incubation, donor and acceptor beads were diluted 1:50 in 150 mM NaCl reaction buffer to a final bead concentration of 20 μg/ml, and 5 μl of each bead dilution was added to each sample, followed by incubation in the dark for 60 min before light emission was measured using the EnVision reader (PerkinElmer) and analyzed using the EnVision manager software.

Analysis of binding curves

The simple binding reaction of protein P interacting with oligonucleotide DNA is described by:

$$P + DNA \xrightleftharpoons{k_d} P \cdot DNA \tag{1}$$

$$K_d = \frac{[P][DNA]}{[P \cdot DNA]} \tag{2}$$

$$[P] = [P]_0 - [P \cdot DNA] \tag{3}$$

$$[DNA] = [DNA]_0 - [P \cdot DNA] \tag{4}$$

where [P] and $[P]_0$ are the concentrations of free protein and total protein, respectively; [DNA] and $[DNA]_0$ are the concentrations of free oligonucleotide and total oligonucleotide, respectively; [P·DNA] is the concentration of the complex; and K_d is the equilibrium dissociation constant. Substituting [DNA] in Eq. (2) with Eq. (4) gives:

$$F_{bound} = \frac{[P \cdot DNA]}{[DNA]_0} = \frac{[P]}{K_d + [P]} \tag{5}$$

If $[DNA]_0 \ll K_d$, then $[P] \approx [P]_0$. Hyperbolic binding curves were fitted to:

$$Signal = Signal_{max} \frac{[P]_0}{K_d + [P]_0} \tag{6}$$

In other cases, [P] in Eq. (5) was substituted with Eq. (3), and the resulting equation was solved for [P·DNA]. Hyperbolic binding curves were then fitted to:

Signal =

$$\frac{[P]_0 + K_d + Signal_{max} - \sqrt{\left([P]_0 + K_d + Signal_{max}\right)^2 - 4[P]_0 Signal_{max}}}{2} \tag{7}$$

The fully cooperative binding reaction of protein P with oligonucleotide DNA is described by:

$$nP + DNA \xrightleftharpoons{k_d} P_n \cdot DNA \tag{8}$$

$$K_d = K_{d,site}^n = \frac{[P]^n[DNA]}{[P_n \cdot DNA]} \tag{9}$$

where K_d is the overall dissociation constant, $K_{d,site}$ is the site dissociation constant, and n is the Hill coefficient describing the stoichiometry of the complex. Substituting [DNA] in Eq. (9) gives the Hill equation:

$$F_{bound} = \frac{[P_n \cdot DNA]}{[DNA]_0} = \frac{[P]^n}{K_{d,site}^n + [P]^n} \tag{10}$$

If $[DNA]_0 \ll K_{d,site}$, then $[P] \approx [P]_0$. Binding curves from AlphaScreen can then be fitted to:

$$\begin{aligned} Signal = {} & Background + \left(Signal_{max} - Background\right) \\ & \times \left(\frac{[P]_0^n}{K_d^n + [P]_0^n}\right) \end{aligned}$$

Binding curves were fitted to GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). To determine IC_{50}, we fitted

the AlphaScreen signal with a log(inhibitor) response curve also using GraphPad/GraphPrism software.

Measurement of IN activities using ELISA

ELISA was performed as previously described to measure both 3′ processing and strand transfer activity.[75] In brief, we used a dsDNA substrate mimicking the viral LTR, consisting of a 3′-end biotin-labeled sense oligonucleotide (5′-ACTGC-TAGAGATTTTCCACACTGACTAAAAGGGTC-biotin; Sigma-Aldrich) and its 5′-end digoxigenin-labeled complement (Sigma-Aldrich). His$_6$–IN was diluted in 10 mM Tris–HCl (pH 7.6), 750 mM NaCl, 10% (vol/vol) glycerol, and 1 mM β-mercaptoethanol. The final reaction mixture (40 μl) was made in reaction buffer [20 mM Hepes (pH 7.5), 75 mM NaCl, 10 mM MgCl$_2$, 5 mM dithiothreitol, 5% (vol/vol) polyethylene glycol 8000, and 15% (vol/vol) dimethylsulfoxide] and contained 70 nM DNA, 450 nM IN, and 200 nM untagged recombinant LEDGF/p75. A 30-min preincubation at 4 °C was followed by the enzymatic reaction at 37 °C for 90 min. After the reaction, an immunosorbent assay was performed on avidin-coated plates (PerkinElmer), as previously described.[75]

Solubility assay

A solubility assay, where the protein concentration in the insoluble fraction *versus* the soluble fraction is compared, was set up to determine the overall protein solubility under AlphaScreen reaction conditions. After protein stock solutions had been thawed, they were centrifuged briefly (14,000 rpm for 1 min) before pipetting to ensure removal of any aggregated protein in the protein preparations. In 30 μl of Hepes/Chaps AlphaScreen buffer, 1.5 μM IN and either 3 μM DNA, 0.5 μM untagged LEDGF/p75, or MBP-LEDGF/p75$_{326–530}$ was added in a 100–500 mM NaCl gradient. After incubation at 4 °C for 1 h, insoluble protein in the samples was pelleted by centrifugation at 14,000 rpm for 1 min. The supernatant was transferred to a new tube, and 10 μl of 6× protein loading dye [160 mM Tris–HCl (pH 6.8), SDS 2%, 200 mM DTT, 40% vol/vol glycerol, and 0.1% wt/vol bromophenol blue] was added. The pellet was resuspended in 20 μl of 6× protein loading dye, and 10 μl of this sample was run in a 12% SDS-PAGE gel in parallel with 20 μl of the soluble fraction. Gels were stained with Coomassie Brilliant Blue® (Acros Organics BVBA, Geel, Belgium). The solubility of IN was also measured at 100 and 150 mM NaCl (Supplementary Fig. 2). Here, the entire fraction of insoluble protein was resuspended in 10 μl of loading buffer and separated by 10% SDS-PAGE in parallel with 10 μl of the corresponding soluble fraction. Proteins were detected by Coomassie stain.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.03.073

References

1. Engelman, A., Mizuuchi, K. & Craigie, R. (1991). HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell*, **67**, 1211–1221.
2. Bushman, F. D. & Craigie, R. (1991). Activities of human immunodeficiency virus (HIV) integration protein *in vitro*: specific cleavage and integration of HIV DNA. *Proc. Natl Acad. Sci. USA*, **88**, 1339–1343.
3. Ciuffi, A. & Bushman, F. D. (2006). Retroviral DNA integration: HIV and the role of LEDGF/p75. *Trends Genet.* **22**, 388–395.
4. Van Maele, B. & Debyser, Z. (2005). HIV-1 integration: an interplay between HIV-1 integrase, cellular and viral proteins. *AIDS Rev.* **7**, 26–43.
5. Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y. *et al.* (2003). HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *J. Biol. Chem.* **278**, 372–381.
6. Engelman, A. & Cherepanov, P. (2008). The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. *PLoS Pathog.* **4**, e1000046.
7. Poeschla, E. M. (2008). Integrase, LEDGF/p75 and HIV replication. *Cell. Mol. Life. Sci.* **65**, 1403–1424.
8. Singh, D. P., Fatma, N., Kimura, A., Chylack, L. T., Jr. & Shinohara, T. (2001). LEDGF binds to heat shock and stress-related element to activate the expression of stress-related genes. *Biochem. Biophys. Res. Commun.* **283**, 943–955.
9. Shinohara, T., Singh, D. P. & Fatma, N. (2002). LEDGF, a survival factor, activates stress-related genes. *Prog. Retinal Eye Res.* **21**, 341–358.
10. Llano, M., Vanegas, M., Hutchins, N., Thompson, D., Delgado, S. & Poeschla, E. M. (2006). Identification and characterization of the chromatin-binding domains of the HIV-1 integrase interactor LEDGF/p75. *J. Mol. Biol.* **360**, 760–773.
11. Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z. & Engelborghs, Y. (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J. Biol. Chem.* **278**, 33528–33539.
12. Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D. *et al.* (2005). Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. *J. Biol. Chem.* **280**, 25517–25523.
13. Shun, M. C., Daigle, J. E., Vandegraaff, N. & Engelman, A. (2007). Wild-type levels of human immunodeficiency virus type 1 infectivity in the absence of cellular emerin protein. *J. Virol.* **81**, 166–172.
14. Busschots, K., Voet, A., De Maeyer, M., Rain, J. C., Emiliani, S., Benarous, R. *et al.* (2007). Identification

of the LEDGF/p75 binding site in HIV-1 integrase. *J. Mol. Biol.* **365**, 1480–1492.

15. Rahman, S., Lu, R., Vandegraaff, N., Cherepanov, P. & Engelman, A. (2007). Structure-based mutagenesis of the integrase–LEDGF/p75 interface uncouples a strict correlation between *in vitro* protein binding and HIV-1 fitness. *Virology*, **357**, 79–90.

16. Vandekerckhove, L., Christ, F., Van Maele, B., De Rijck, J., Gijsbers, R., Van den Haute, C. *et al.* (2006). Transient and stable knockdown of the integrase cofactor LEDGF/p75 reveals its role in the replication cycle of human immunodeficiency virus. *J. Virol.* **80**, 1886–1896.

17. Llano, M., Saenz, D. T., Meehan, A., Wongthida, P., Peretz, M., Walker, W. H. *et al.* (2006). An essential role for LEDGF/p75 in HIV integration. *Science*, **314**, 461–464.

18. Marshall, H. M., Ronen, K., Berry, C., Llano, M., Sutherland, H., Saenz, D. *et al.* (2007). Role of PSIP1/LEDGF/p75 in lentiviral infectivity and integration targeting. *PLoS One*, **2**, e1340.

19. Turlure, F., Maertens, G., Rahman, S., Cherepanov, P. & Engelman, A. (2006). A tripartite DNA-binding element, comprised of the nuclear localization signal and two AT-hook motifs, mediates the association of LEDGF/p75 with chromatin *in vivo*. *Nucleic Acids Res.* **34**, 1653–1675.

20. Llano, M., Vanegas, M., Hutchins, N., Thompson, D., Delgado, S. & Poeschla, E. M. (2006). Identification and characterization of the chromatin-binding domains of the HIV-1 integrase interactor LEDGF/p75. *J. Mol. Biol.* **360**, 760–773.

21. Cherepanov, P., Ambrosio, A. L., Rahman, S., Ellenberger, T. & Engelman, A. (2005). Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. *Proc. Natl. Acad. Sci. USA*, **102**, 17308–17313.

22. Cherepanov, P., Devroe, E., Silver, P. A. & Engelman, A. (2004). Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. *J. Biol. Chem.* **279**, 48883–48892.

23. Cherepanov, P., Sun, Z. Y., Rahman, S., Maertens, G., Wagner, G. & Engelman, A. (2005). Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75. *Nat. Struct. Mol. Biol.* **12**, 526–532.

24. Yang, J. & Everett, A. D. (2007). Hepatoma-derived growth factor binds DNA through the N-terminal PWWP domain. *BMC Mol. Biol.* **8**, 101.

25. Botbol, Y., Raghavendra, N. K., Rahman, S., Engelman, A. & Lavigne, M. (2008). Chromatinized templates reveal the requirement for the LEDGF/p75 PWWP domain during HIV-1 integration *in vitro*. *Nucleic Acids Res.* **36**, 1237–1246.

26. Gijsbers, R., Ronen, K., Vets, S., Malani, N., De Rijck, J., McNeely, M. *et al.* (2010). LEDGF hybrids efficiently retarget lentiviral integration into heterochromatin. *Mol. Ther.* **18**, 552–560.

27. Ciuffi, A., Diamond, T. L., Hwang, Y., Marshall, H. M. & Bushman, F. D. (2006). Modulating target site selection during human immunodeficiency virus DNA integration *in vitro* with an engineered tethering factor. *Hum. Gene. Ther.* **17**, 960–967.

28. Shun, M. C., Raghavendra, N. K., Vandegraaff, N., Daigle, J. E., Hughes, S., Kellam, P. *et al.* (2007). LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes Dev.* **21**, 1767–1778.

29. De Rijck, J., Bartholomeeusen, K., Ceulemans, H., Debyser, Z. & Gijsbers, R. (2010). High-resolution profiling of the LEDGF/p75 chromatin interaction in the ENCODE region. *Nucleic Acids Res.* **38**, 6135–6147.

30. Busschots, K., Vercammen, J., Emiliani, S., Benarous, R., Engelborghs, Y., Christ, F. & Debyser, Z. (2005). The interaction of LEDGF/p75 with integrase is lentivirus-specific and promotes DNA binding. *J. Biol. Chem.* **280**, 17841–17847.

31. Yu, F., Jones, G. S., Hung, M., Wagner, A. H., MacArthur, H. L., Liu, X. *et al.* (2007). HIV-1 integrase preassembled on donor DNA is refractory to activity stimulation by LEDGF/p75. *Biochemistry*, **46**, 2899–2908.

32. Raghavendra, N. K. & Engelman, A. (2007). LEDGF/p75 interferes with the formation of synaptic nucleoprotein complexes that catalyze full-site HIV-1 DNA integration *in vitro*: implications for the mechanism of viral cDNA integration. *Virology*, **360**, 1–5.

33. Pandey, K. K., Sinha, S. & Grandgenett, D. P. (2007). Transcriptional coactivator LEDGF/p75 modulates human immunodeficiency virus type 1 integrase-mediated concerted integration. *J. Virol.* **81**, 3969–3979.

34. Cherepanov, P., Devroe, E., Silver, P. A. & Engelman, A. (2004). Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. *J. Biol. Chem.* **279**, 48883–48892.

35. McKee, C. J., Kessl, J. J., Shkriabai, N., Dar, M. J., Engelman, A. & Kvaratskhelia, M. (2008). Dynamic modulation of HIV-1 integrase structure and function by cellular lens epithelium-derived growth factor (LEDGF) protein. *J. Biol. Chem.* **283**, 31802–31812.

36. Gupta, K., Diamond, T., Hwang, Y., Bushman, F. & Van Duyne, G. D. (2010). Structural properties of HIV integrase–LEDGF oligomers. *J. Biol. Chem.* **285**, 20303–20315.

37. Kotova, S., Li, M., Dimitriadis, E. K. & Craigie, R. (2010). Nucleoprotein intermediates in HIV-1 DNA integration visualized by atomic force microscopy. *J. Mol. Biol.* **399**, 491–500.

38. De Rijck, J., Vandekerckhove, L., Gijsbers, R., Hombrouck, A., Hendrix, J., Vercammen, J. *et al.* (2006). Overexpression of the lens epithelium-derived growth factor/p75 integrase binding domain inhibits human immunodeficiency virus replication. *J. Virol.* **80**, 11498–11509.

39. Hombrouck, A., De Rijck, J., Hendrix, J., Vandekerckhove, L., Voet, A., De Maeyer, M. *et al.* (2007). Virus evolution reveals an exclusive role for LEDGF/p75 in chromosomal tethering of HIV. *PLoS Pathog.* **3**, e47.

40. van Gent, D. C., Elgersma, Y., Bolk, M. W., Vink, C. & Plasterk, R. H. (1991). DNA binding properties of the integrase proteins of human immunodeficiency viruses types 1 and 2. *Nucleic Acids Res.* **19**, 3821–3827.

41. Hazuda, D. J., Felock, P. J., Hastings, J. C., Pramanik, B. & Wolfe, A. L. (1997). Differential divalent cation requirements uncouple the assembly and catalytic reactions of human immunodeficiency virus type 1 integrase. *J. Virol.* **71**, 7005–7011.

42. Woerner, A. M., Klutch, M., Levin, J. G. & Marcus-Sekura, C. J. (1992). Localization of DNA binding activity of HIV-1 integrase to the C-terminal half of the protein. *AIDS Res. Hum. Retroviruses*, **8**, 297–304.

43. Vink, C., Oude Groeneger, A. M. & Plasterk, R. H. (1993). Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type I integrase protein. *Nucleic Acids Res.* **21**, 1419–1425.

44. Khan, E., Mack, J. P., Katz, R. A., Kulkosky, J. & Skalka, A. M. (1991). Retroviral integrase domains: DNA binding and the recognition of LTR sequences. *Nucleic Acids Res.* **19**, 851–860.

45. Sherman, P. A. & Fyfe, J. A. (1990). Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. *Proc. Natl Acad. Sci. USA*, **87**, 5119–5123.

46. Mazumder, A., Cooney, D., Agbaria, R., Gupta, M. & Pommier, Y. (1994). Inhibition of human immunodeficiency virus type 1 integrase by 3′-azido-3′-deoxythymidylate. *Proc. Natl Acad. Sci. USA*, **91**, 5771–5775.

47. Haugan, I. R., Nilsen, B. M., Worland, S., Olsen, L. & Helland, D. E. (1995). Characterization of the DNA-binding activity of HIV-1 integrase using a filter binding assay. *Biochem. Biophys. Res. Commun.* **217**, 802–810.

48. Lutzke, R. A., Vink, C. & Plasterk, R. H. (1994). Characterization of the minimal DNA-binding domain of the HIV integrase protein. *Nucleic Acids Res.* **22**, 4125–4131.

49. Drelich, M., Haenggi, M. & Mous, J. (1993). Conserved residues Pro-109 and Asp-116 are required for interaction of the human immunodeficiency virus type 1 integrase protein with its viral DNA substrate. *J. Virol.* **67**, 5041–5044.

50. Engelman, A., Hickman, A. B. & Craigie, R. (1994). The core and carboxyl-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to nonspecific DNA binding. *J. Virol.* **68**, 5911–5917.

51. Pemberton, I. K., Buc, H. & Buckle, M. (1998). Displacement of viral DNA termini from stable HIV-1 integrase nucleoprotein complexes induced by secondary DNA-binding interactions. *Biochemistry*, **37**, 2682–2690.

52. Cherepanov, P., Este, J. A., Rando, R. F., Ojwang, J. O., Reekmans, G., Steinfeld, R. *et al.* (1997). Mode of interaction of G-quartets with the integrase of human immunodeficiency virus type 1. *Mol. Pharmacol.* **52**, 771–780.

53. Vaisocherova, H., Snasel, J., Springer, T., Sipova, H., Rosenberg, I., Stepanek, J. & Homola, J. (2009). Surface plasmon resonance study on HIV-1 integrase strand transfer activity. *Anal. Bioanal. Chem.* **393**, 1165–1172.

54. Vercammen, J., Maertens, G., Gerard, M., De Clercq, E., Debyser, Z. & Engelborghs, Y. (2002). DNA-induced polymerization of HIV-1 integrase analyzed with fluorescence fluctuation spectroscopy. *J. Biol. Chem.* **277**, 38045–38052.

55. Delelis, O., Carayon, K., Guiot, E., Leh, H., Tauc, P., Brochon, J. C. *et al.* (2008). Insight into the integrase–DNA recognition mechanism. A specific DNA-binding mode revealed by an enzymatically labeled integrase. *J. Biol. Chem.* **283**, 27838–27849.

56. Guiot, E., Carayon, K., Delelis, O., Simon, F., Tauc, P., Zubin, E. *et al.* (2006). Relationship between the oligomeric status of HIV-1 integrase on DNA and enzymatic activity. *J. Biol. Chem.* **281**, 22707–22719.

57. Carayon, K., Leh, H., Henry, E., Simon, F., Mouscadet, J. F. & Deprez, E. A cooperative and specific DNA-binding mode of HIV-1 integrase depends on the nature of the metallic cofactor and involves the zinc-containing N-terminal domain. Nucleic Acids Res. 38, 3692–3708.

58. Pemberton, I. K., Buckle, M. & Buc, H. (1996). The metal ion-induced cooperative binding of HIV-1 integrase to DNA exhibits a marked preference for Mn(II) rather than Mg(II). *J. Biol. Chem.* **271**, 1498–1506.

59. Yi, J., Asante-Appiah, E. & Skalka, A. M. (1999). Divalent cations stimulate preferential recognition of a viral DNA end by HIV-1 integrase. *Biochemistry*, **38**, 8458–8468.

60. Deprez, E., Tauc, P., Leh, H., Mouscadet, J. F., Auclair, C. & Brochon, J. C. (2000). Oligomeric states of the HIV-1 integrase as measured by time-resolved fluorescence anisotropy. *Biochemistry*, **39**, 9275–9284.

61. Deprez, E., Barbe, S., Kolaski, M., Leh, H., Zouhiri, F., Auclair, C. *et al.* (2004). Mechanism of HIV-1 integrase inhibition by styrylquinoline derivatives *in vitro*. *Mol. Pharmacol.* **65**, 85–98.

62. Faure, A., Calmels, C., Desjobert, C., Castroviejo, M., Caumont-Sarcos, A., Tarrago-Litvak, L. *et al.* (2005). HIV-1 integrase crosslinked oligomers are active *in vitro*. *Nucleic Acids Res.* **33**, 977–986.

63. Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M. & Poeschla, E. M. (2004). LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. *J. Virol.* **78**, 9524–9537.

64. Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z. & Engelborghs, Y. (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J. Biol. Chem.* **278**, 33528–33539.

65. Hendrix, J., Gijsbers, R., De Rijck, J., Voet, A., Hotta, J. I., McNeely, M. *et al.* (2011). The transcriptional co-activator LEDGF/p75 displays a dynamic scan-and-lock mechanism for chromatin tethering. *Nucleic Acids Res.* **39**, 1310–1325.

66. Hare, S., Shun, M. C., Gupta, S. S., Valkov, E., Engelman, A. & Cherepanov, P. (2009). A novel co-crystal structure affords the design of gain-of-function lentiviral integrase mutants in the presence of modified PSIP1/LEDGF/p75. *PLoS Pathog.* **5**, e1000259.

67. Hare, S. & Cherepanov, P. (2009). The interaction between lentiviral integrase and LEDGF: structural and functional insights. *Viruses*, **1**, 780–801.

68. Hare, S., Di Nunzio, F., Labeja, A., Wang, J., Engelman, A. & Cherepanov, P. (2009). Structural basis for functional tetramerization of lentiviral integrase. *PLoS Pathog.* **5**, e1000515.

69. Turlure, F., Maertens, G., Rahman, S., Cherepanov, P. & Engelman, A. (2006). A tripartite DNA-binding element, comprised of the nuclear localization signal and two AT-hook motifs, mediates the association of LEDGF/p75 with chromatin *in vivo*. *Nucleic Acids Res.* **34**, 1653–1665.

70. Tsiang, M., Jones, G. S., Hung, M., Mukund, S., Han, B., Liu, X. *et al.* (2009). Affinities between the binding partners of the HIV-1 integrase dimer–lens epithelium-derived growth factor (IN dimer–LEDGF) complex. *J. Biol. Chem.* **284**, 33580–33599.

71. Maertens, G., Vercammen, J., Debyser, Z. & Engelborghs, Y. (2005). Measuring protein–protein interactions inside living cells using single color fluorescence correlation spectroscopy. Application to human immunodeficiency virus type 1 integrase and LEDGF/p75. *FASEB J.* **19**, 1039–1041.

72. Chiu, J., March, P. E., Lee, R. & Tillett, D. (2004). Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res.* **32**, e174.

73. Bartholomeeusen, K., De Rijck, J., Busschots, K., Desender, L., Gijsbers, R., Emiliani, S. *et al.* (2007). Differential interaction of HIV-1 integrase and JPO2 with the C terminus of LEDGF/p75. *J. Mol. Biol.* **372**, 407–421.

74. Kirsch, R. D. & Joly, E. (1998). An improved PCR-mutagenesis strategy for two-site mutagenesis or sequence swapping between related genes. *Nucleic Acids Res.* **26**, 1848–1850.

75. Hwang, Y., Rhodes, D. & Bushman, F. (2000). Rapid microtiter assays for poxvirus topoisomerase, mammalian type IB topoisomerase and HIV-1 integrase: application to inhibitor isolation. *Nucleic Acids Res.* **28**, 4884–4892.