HIV-1 Integrase Preassembled on Donor DNA Is Refractory to Activity Stimulation by LEDGF/p75

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ABSTRACT: LEDGF/p75 is known to enhance the integrase strand transfer activity in vitro, but the underlying mechanism is unclear. Using an integrase assay with a chemiluminescent readout adapted to a 96-well plate format, the effect of LEDGF/p75 on both the 3′-processing and strand transfer steps was analyzed. Integrase inhibitors of the strand transfer reaction remained active in the presence of LEDGF/p75, but displayed 3- to 7-fold higher IC_{50} values. Our analyses indicate that, in the presence of 150 nM LEDGF/p75, active integrase/donor DNA complexes were increased by 5.3-fold during the 3′-processing step. In addition, these integrase/donor DNA complexes showed a 4.5-fold greater affinity for the target DNA during the subsequent strand transfer step. We also observed a 3.7-fold increase in the rate constant of catalysis of the strand transfer step when 150 nM LEDGF/p75 was present during the 3′-processing step. In contrast, when LEDGF/p75 was added at the beginning of the strand transfer step, no increase in either the concentration of active integrase/donor DNA complex or its rate constant of strand transfer catalysis was observed. This observation suggested that the integrase/donor DNA formed in the absence of LEDGF/p75 became refractory to the stimulatory effect of LEDGF/p75. Instead, this LEDGF/p75 added at the start of the strand transfer step was able to promote the formation of a new cohort of active integrase/donor DNA complexes which became functional with a delay of 45 min after LEDGF/p75 addition. We propose a model whereby LEDGF/p75 can only bind integrase before the latter binds donor DNA whereas donor DNA can engage either free or LEDGF/p75-bound integrase.

HIV-1 integrase is a 32 kDa protein essential for viral replication and responsible for the integration of double stranded viral DNA, the product of reverse transcription, into host chromosomal DNA. Numerous biochemical and genetic analyses indicate that integrase can form multimers (*1*−*5*), however the functional intracellular oligomeric status of integrase remains unknown. In cells, integrase functions within a preintegration complex and possibly exists in a multimeric form that could be stabilized by the presence of viral (*6*, *7*) and host (*8*−*14*) factors and nucleic acid components.

Recently, the lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75[1]) has been identified as the dominant binding partner of HIV-1 integrase in human cells (*15*−*19*). In vitro, LEDGF/p75 was shown to interact specifically with lentivirus integrases and to enhance the strand transfer activity, solubility, and DNA-binding ability of HIV-1 integrase (*15*, *20*, *21*). In cells, LEDGF/p75 is a component of lentiviral preintegration complexes, is essential for the nuclear and chromosomal targeting of HIV-1 integrase through a functional nuclear localization signal and a chromatin tethering domain (*16*, *18*, *22*, *23*), and represents the first example of a host factor controlling the integration sites of HIV-1 in human cells (*24*). An evolutionarily conserved 83-residue domain located in the C-terminal region of LEDGF/p75 was identified as the integrase binding domain (IBD), the solution structure of which has been determined (*20*, *23*, *25*). On integrase, several type II mutations that disrupted the interaction with LEDGF/p75 have also been reported (*25*−*27*). More recently, the crystal structure of the catalytic core dimer of HIV-1 integrase complexed with the IBD of LEDGF/p75 has been solved, revealing a binding pocket for the IBD of LEDGF/p75 between two integrase monomers (*28*). Through transient and stable knockdown of LEDGF/p75 and overexpression of the IBD in target cells, it has now become clear that LEDGF/p75 is an important cellular cofactor for HIV-1 replication (*29*−*31*).

To further understand the mechanism by which LEDGF/p75 interacts with integrase to enhance its strand transfer activity, we used a 96-well plate-adapted integrase assay with a chemiluminescent readout to study the kinetics of 3′-processing and strand transfer in the presence and absence of LEDGF/p75. The study dissected the effect of LEDGF/p75 on the various kinetic parameters of HIV-1 integrase and revealed a possible sequence of interactions between integrase, LEDGF/p75, and donor DNA.

EXPERIMENTAL PROCEDURES

Construction of 6His-Tagged LEDGF/p75. The sequence of LEDGF/p75 was amplified from total RNA isolated from

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[1] LEDGF/p75, lens epithelium-derived growth factor/transcription coactivator p75; IBD, integrase binding domain of LEDGF/p75; DEPC, diethyl pyrocarbonate; IPTG, isopropyl β-D-thiogalactopyranoside; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate buffer; RLU, relative luminescence unit; CCD, catalytic core domain of integrase.

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fresh HeLa cells grown to ~80% confluence using RNA STAT-60 cell lysis reagent (Tel-Test "B", Inc., Friendswood, TX). Total RNA was isolated according to the manufacturer's protocol. The final RNA pellet was dissolved in 60 μL of DEPC treated TE buffer and stored at −80 °C. The LEDGFp75 sequence was cloned either as an N-terminal 6His-tagged protein or as a C-terminal 6His-tagged protein. The RT-PCR amplification/cloning primers are as follows: *Nde*I/p75N5′primer1, 5′ATATACATATGCATCACCATCACCATCACTCTGGT**ACTCGCGATTT CAAACCTGG** 3′, and p75N/*Hin*dIII3′primer, 5′-AGCCGGATCAAGCTT**CTAGTTATCTAGTGT AGAATCC**-3′ for the N-terminal 6His-tagged protein; and *Nde*I/p75C5′primer, 5′-ATATACAT **ATGACTCGCGATTTCAAACCTGG**-3′, and p75C/*Hin*dIII3′primer1, 5′AGCCGGATCAAGCTTCTAGTGATGGTGATGGTGATGAGACC**GTTATCTAGTGTAGAATCCTTC**-3′ for the C-terminal 6His-tagged protein. After RT-PCR amplification, the amplification products were purified using the QIAquick PCR purification kit (catalog #28104, QIAGEN, Valencia, CA) and digested with *Nde*I and *Hin*dIII. The *Nde*I and *Hin*dIII cut inserts were purified on a 1% agarose gel and cloned into the *Nde*I and *Hin*dIII sites of the T7 expression vector, pRSET B (catalog #V351-20, Invitrogen, Carlsbad, CA). The N-terminal 6His-tagged construct is named p6His-LEDGF75, and the C-terminal 6His-tagged construct is named pLEDGF75-6His. Both constructs were introduced into *Escherichia coli* BL21-Gold(DE3)pLysS (catalog #230134, Stratagene, La Jolla, CA) for protein expression.

Expression of LEDGF/p75. For protein expression, an overnight culture of BL21-Gold(DE3)pLysS transformed with an LEDGFp75 expression construct and grown at 37 °C and 250 rpm was used to inoculate 6 liters of L-broth containing ampicillin and chloramphenicol at an initial density of 0.05 OD_{600}/mL. The culture was grown at 37 °C and 250 rpm until a density of $OD_{600} = 0.8-1.0$ was reached in 3−4 h. Protein expression was then induced by addition of IPTG to a final concentration of 1 mM. The culture was kept at 30 °C and 250 rpm for 3 additional hours before the cells were pelleted and stored at −80 °C. Sixteen grams of cell pellet was resuspended in 200 mL of Lysis buffer (50 mM Tris pH 7.6, 300 mM NaCl, 5% glycerol, 0.5% NP40), sonicated and lysed by passing three times through a microfluidizer at 1500 psi (Microfluidics, Newton, MA). Insoluble material was removed by centrifugation at 17500*g* for 60 min at 4 °C, and the supernatant (S1) was saved for protein purification using a three-column method.

Purification of LEDGF/p75. The supernatant (S1) from the previous section was loaded at 5 mL/min onto a 10 mL Ni chelating affinity column (HisTrap HP, catalog #17-5248-02, Amersham Biosciences Corp, Piscataway, NJ) pre-equilibrated with Ni buffer A (50 mM Tris pH 7.6, 300 mM NaCl, 5 mM imidazole-HCl, 10% glycerol, 2 mM β-mercaptoethanol). The column was washed with 10 column volumes of Ni buffer A, and bound protein was eluted with 20 column volumes of imidazole gradient (5−500 mM) at 5 mL/min. Three milliliter fractions were collected during the elution. Column fractions were analyzed by SDS−PAGE (NuPAGE 4−12% Bis-Tris gel, catalog #NP0322BOX, Invitrogen, Carlsbad, CA). Fractions containing the ~75 kDa LEDGF/p75 band (eluting at ~100−125 mM imidazole) were pooled, diluted 5-fold with SP buffer A (10 mM $NaPO_4$ pH 6.5, 10% glycerol, 1 mM DTT), adjusted to pH 6.5, and loaded onto a 5 mL cation exchange column (HiTrap SP FF column, catalog #17-5157-01, Amersham Biosciences Corp, Piscataway, NJ) pre-equilibrated with the same buffer. The column was washed with 2 column volumes of SP buffer A, and bound protein was eluted with 25 column volumes of NaCl gradient (0−1000 mM) at 3 mL/min. Column fractions were analyzed by SDS−PAGE, and fractions containing the LEDGF/p75 band were pooled and concentrated to 1 mL (Centriprep-30, catalog #4322, Millipore, Billerica, MA) and loaded onto a 100 mL gel filtration column (2.5 × 30 cm KW2003, catalog #KW2003, Showa Denko KK, Japan) pre-equilibrated with 10 mM $NaPO_4$ pH 6.5, 10% glycerol, 500 mM NaCl, 1 mM DTT. Fractions containing LEDGF/p75, corresponding to a molecular weight of ~75 kDa, were pooled, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C. The yields of LEDGF/p75-6His and 6His-LEDGF/p75 are 0.6 mg/g and 0.1 mg/g bacterial pellet, respectively. Purity of LEDGF/p75 was >90% by SDS−PAGE. The final concentrations of LEDGF/p75-6His and 6His-LEDGF/p75 were 1.5 and 1.2 mg/mL, respectively.

Standard Integrase Strand Transfer Assay. This assay measures the 3′-end joining activity of integrase which nicks one strand of the target DNA and ligates the 3′-processed end of the donor DNA to the 5′-end of the target DNA at the nick. One hundred microliters per well of 0.14 μM biotinylated donor DNA was first bound to Reacti-Bind High Binding Capacity Streptavidin coated white plates (catalog #15502, Pierce, Rockford, IL) for 1 h at room temperature. After washing away unbound donor DNA with PBS, 80 μL of integrase mixture containing (25 mM HEPES pH 7.3, 12.5 mM DTT, 93.75 mM NaCl, 12.5 mM $MgCl_2$, 1.25% glycerol, 0.3 μM integrase) was added to each well to allow 3′-processing of the donor DNA end for 30 min at 37 °C. Ten microliters of serially 2.2-fold diluted compound (9 concentration points) and 10 μL of DIG-tagged target DNA were then sequentially added to each well and the 3′-end-joining reaction was allowed to proceed for 30 min at 37 °C. The final reaction mixture contained 20 mM HEPES pH 7.3, 10 mM DTT, 75 mM NaCl, 10 mM $MgCl_2$, 1% glycerol, 0.25 μM integrase, 0.25 μM target DNA, ~7 pmol of plate-bound donor DNA and ± compound. The wells were then washed three times with 2X SSC (catalog #S-6639, Sigma, St Louis, MO) to remove unjoined target DNA. One hundred microliters of 2000-fold diluted HRP-conjugated Anti-DIG antibody (catalog #31468, Pierce, Rockford, IL) was added to bind the DIG-tag of the 3′-end-joined target DNA for 1 h at room temperature. After washing away unbound antibody, 100 μL of SuperSignal ELISA Femto Substrate working solution (catalog #37075, Pierce, Rockford, IL) was added and chemiluminescence was read immediately at 425 nm. The chemiluminescent readout for the inhibitor dose response was analyzed by curve fitting with eq I after conversion of the data to % uninhibited to determine the 50% inhibitory concentration, IC_{50},

$$y = \frac{100 \times IC_{50}{}^{n}}{IC_{50}{}^{n} + [I]^{n}} \quad \text{(I)}$$

where y = % uninhibited, n = Hill coefficient, and [I] = inhibitor concentration.

The two strands of the unprocessed donor DNA are as follows: LTR5′Biotin, 5′Biotin-ACCCTTTTAGTCAGT GTGGAAAATCTCTAGCAGT-3′, and LTR2, 3′-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5′. The two strands of the DIG-tagged target DNA are as follows: TargetDIG1, 5′-TGACCAAGGGCTAATTCACT-3′DIG, and Target DIG2, 3′DIG-ACTGGTTCCCGATTAAGTGA-5′. For 3′-processed donor DNA, the biotinylated strand is substituted with LTRP5′Biotin: 5′Biotin-ACCCTTTTAGTCAGTGTG GAAAATCTCT AGCA-3′.

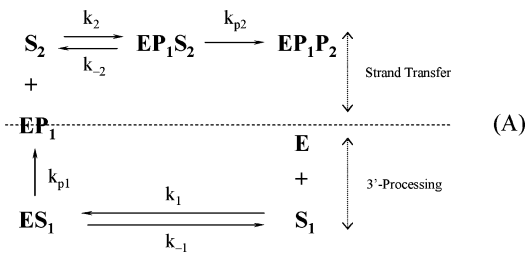
LEDGF/p75 Strand Transfer Activity Enhancement Assay. To measure integrase activity enhancement by LEDGF/p75, LEDGF/p75 was added either simultaneously with integrase to donor DNA coated wells before 3′-processing or simultaneously with target DNA after 3′-processing. For strand transfer time course assays, the 3′-end joining reaction was allowed to proceed for a desired time after which the content of the well is removed and replaced with 100 μL of 2X SSC. After completion of the time course, all the wells were washed with 2X SSC and further processed as described in the standard strand transfer assay.

Analysis of LEDGF/p75 Dose Response. The strand transfer activity data from an LEDGF/p75 dose response were converted to fold-enhancement over activity measured in the absence of LEDGF/p75 and analyzed with eq II by curve fitting,

$$y = M - \frac{(M-1) \times EC_{50}{}^{n}}{EC_{50}{}^{n} + [L]^{n}} \quad \text{(II)}$$

where y = fold-enhancement, M = maximum fold-enhancement ($M > 1$), n = Hill coefficient, [L] = LEDGF/p75 concentration, and EC_{50} = effective concentration of LEDGF/p75 giving half-maximal enhancement.

Analysis of the Strand Transfer Kinetics. The in vitro integrase reaction can be described by scheme A, where S_1 = donor DNA, S_2 = target DNA, P_1 = 3′-processed donor DNA, EP_1 = active integrase/donor DNA complex, and P_1P_2 = integration product. In scheme A we have assumed a tight association of integrase with the strand transfer product as complex EP_1P_2, as for many closely related transposition type reactions, integrase can remain tightly associated with the product. Since 3′-processing was carried out in the absence of S_2 for 90 min prior to the strand transfer time course study when it was shown to be essentially complete (see Supporting Information, Figure S1), the strand transfer step can be treated with classical Michaelis−Menten kinetics where k_{p2} = the rate constant of strand transfer catalysis and k_{-2}/k_2 = the dissociation constant of S_2 from EP_1.



(A)

When S_2 is large relative to EP_1 which does not get easily replenished and assuming P_1P_2 formation is the rate-limiting step, the strand transfer reaction can be simplified as scheme B and treated with first-order kinetics with a rate constant k closely reflecting the rate constant of catalysis, k_{p2}.

$$\underset{(S_0)}{EP_1} \xrightarrow{k} EP_1P_2 \quad \text{(B)}$$

The time course of strand transfer is then described by eq III, where P = concentration of P_1P_2 at any time t and S_0 = initial concentration of EP_1. Using this analysis, the plateau of the strand transfer time course will reflect the initial concentration of the of the active integrase/donor DNA complex.

$$P = S_0(1 - e^{-kt}) \quad \text{(III)}$$

If the time course starts with a delay of t_0, then eq III can be rewritten as eq IV.

$$P = S_0(1 - e^{-k(t-t_0)}) \quad \text{(IV)}$$

The velocity plot of the strand transfer reaction was analyzed using the Michaelis−Menten equation (V), where [S] = target DNA concentration.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(V)}$$

RESULTS

Optimization of the Coating Density of Donor DNA. In order to determine the optimal coating concentration of donor DNA for the strand transfer assay, biotinylated donor DNA at various concentrations up to 2 μM was bound to the streptavidin coated wells. The molar amount of donor DNA bound to the wells was determined by calculating the difference between the OD_{260} of the DNA solution in each well before and after 1 h of coating. The molar amount of bound donor DNA increased with the coating concentration and saturated at ∼58 pmol/well (Figure 1A). The coated wells were used in the strand transfer assay as described in Experimental Procedures, and the strand transfer activity was plotted against the coating concentration (Figure 1A). The strand transfer activity peaked at a donor DNA coating concentration of 0.14 μM, corresponding to 7 pmol of donor DNA bound to the well or 70 nM in a 100 μL reaction (Figure 1A). This represents the maximum potential concentration of active integrase/donor DNA complex that can be assembled if integrase were used in large excess. When the integrase concentration was increased from 0.25 μM used in the standard strand transfer assay to 1.4 μM, the activity increased by ∼15-fold and began to plateau (Figure 1B). This suggests that less than 1/15 (<5 nM) of the bound donor DNA actually gets assembled as active integrase/donor DNA complex under standard strand transfer assay conditions.

LEDGF/p75 Enhances the Strand Transfer Activity of Integrase. Both C- and N-terminally 6His-tagged purified recombinant LEDGF/p75 proteins have >90% purity on NuPAGE 4−12% Bis-Tris gel and migrated with a molecular weight of ∼75 kDa (Figure 2A). To assess the ability of LEDGF/p75 to enhance the strand transfer activity of integrase, increasing concentrations of LEDGF/p75 were added simultaneously with a fixed concentration of integrase to allow 3′-processing for 1.5 h. Target DNA was then added

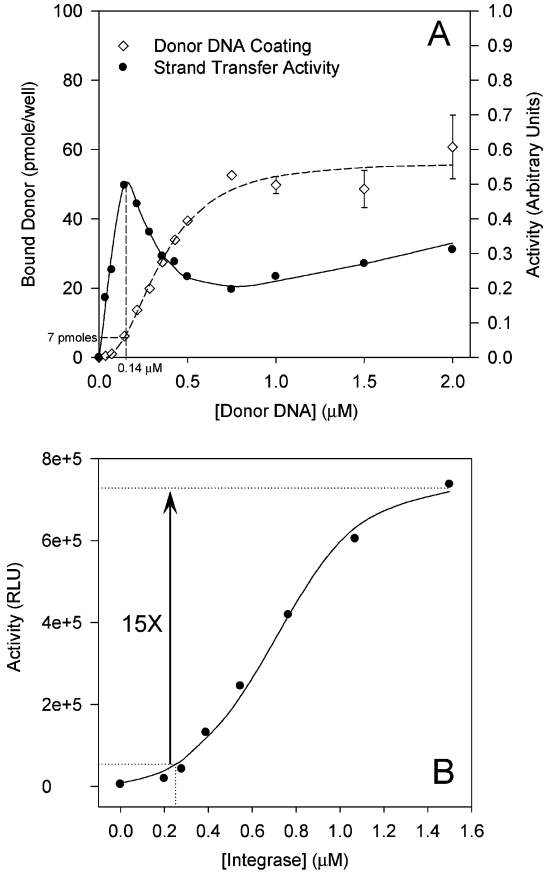


FIGURE 1: Strand transfer assay. (A) Donor DNA dose response of plate coating and strand transfer activity. The molar amount of donor DNA bound to the wells was determined by calculating the difference between the OD_{260} of the DNA solution in each well before and after 1 h of coating. Standard strand transfer assay conditions (i.e., 30 min 3′-processing at 37 °C followed by 30 min 3′-end-joining at 37 °C after addition of target DNA) were used except that integrase was added to the reaction at a final concentration of 0.75 μM. (B) Integrase dose response of strand transfer activity. Standard strand transfer assay conditions were used with varying concentrations of integrase.

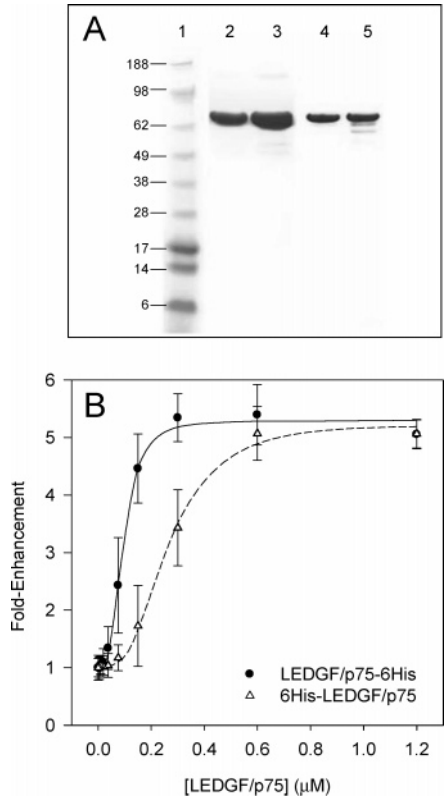


FIGURE 2: Purified recombinant LEDGF/p75. (A) Gel electrophoresis of purified recombinant LEDGF/p75: lanes 2 and 3, peak gel filtration fractions of LEDGF/p75-6His; lanes 4 and 5, peak gel filtration fractions of 6His-LEDGF/p75. (B) LEDGF/p75 dose response of strand transfer enhancement. Standard strand transfer assay conditions were used except that the duration of 3′-processing was 1.5 h. LEDGF/p75 was added before 3′-processing. Data points shown as mean of three independent experiments done in duplicate. The error bars represent the SD of the three experiments. LEDGF/p75-6His, EC_{50} = 93 nM, maximum enhancement = 5.3-fold; 6His-LEDGF/p75, EC_{50} = 264 nM, maximum enhancement = 5.2-fold.

to allow strand transfer for 30 min. Both LEDGF/p75-6His and 6His-LEDGF/p75 were capable of enhancing the strand transfer activity by a maximum of ~5.2-fold (Figure 2B). LEDGF/75-6His has an EC_{50} of 0.093 μM and appears to be a more effective enhancer of strand transfer activity than 6His-LEDGF/p75, which has a 2.8-fold larger EC_{50} of 0.264 μM. LEDGF/p75-6His was used in all subsequent experiments.

Integrase Specific Inhibitors Remain Active against LEDGF/p75-Enhanced Strand Transfer Activity. Three previously reported integrase inhibitors: a diketo acid (GS-16405) (*32*), a diketo triazole (S-1360) (*33*), and a naphthyridine (L-870,810) (*34*) were tested in the strand transfer assay in the absence or presence of 0.15 μM LEDGF/p75 (Table 1). LEDGF/p75 was added to the reaction either before 3′-processing or after 3′-processing. Using a strand transfer time of 60 min, LEDGF/p75 enhanced the strand transfer activity 4.7-fold and 2.5-fold when added before and after 3′-processing respectively (Table 1). In the presence of LEDGF/p75, the three integrase specific inhibitors were still capable of blocking the strand transfer activity with submicromolar concentrations but with increased IC_{50} values whether LEDGF/p75 was added before (3−7-fold increase) or after (1.5−6-fold increase) 3′-processing (Table 1). For competitive inhibitors, such as these integrase strand transfer inhibitors (*35*, *36*), an increase in IC_{50} can be due to an increase in the substrate concentration, [S], a decrease in the K_m of the substrate, or an increase in the K_i of the inhibitor according to eq VI.

$$IC_{50} = \left(1 + \frac{[S]}{K_m}\right)K_i \qquad \text{(VI)}$$

There are two substrates in the strand transfer reaction, the target DNA and the integrase/donor DNA complex. Since target DNA was kept constant in this inhibition study, the relevant substrate was represented by the active integrase/donor DNA complex. In the remaining part of the results, we will assess the ability of LEDGF/p75 to either increase the concentration of active integrase/donor DNA complex or decrease the K_m of target DNA.

LEDGF/p75 Played a Role in the Assembly of Active Integrase/Donor DNA Complex. If LEDGF/p75 increased the concentration of active integrase/donor DNA complex, it had to occur during the 3′-processing step of the assay. An LEDGF/p75 dose response study using nonprocessed donor DNA was carried out with three different durations of 3′-processing (30, 90, and 180 min) while keeping the duration of the strand transfer step at 30 min (Figure 3). When

Table 1: Integrase Inhibitors Remain Active in the Presence of LEDGF/p75[a]

		LEDGF/p75 added before 3′-processing		LEDGF/p75 added after 3′-processing	
compound	IN alone IC_{50} (nM)	IN + LEDGF/p75 IC_{50} (nM)	fold-increase	IN + LEDGF/p75 IC_{50} (nM)	fold-increase
S-1360	130 ± 40	910 ± 190	7.0	830 ± 250	6.4
L-870,810	76 ± 19	310 ± 20	4.1	140 ± 30	1.8
GS-16405	62 ± 16	210 ± 20	3.4	82 ± 15	1.3

[a] The IC_{50} values were determined from a 9-point inhibitor concentration dose response curve using eq I. The errors shown represent the standard error of the fit. The concentrations used are [integrase] = 0.25 μM, [LEDGF/p75] = 0.15 μM, [target DNA] = 250 nM. 3′-Processing time was 30 min, and strand transfer time was 60 min. Adding LEDGF/p75 before 3′-processing gave a 4.7-fold strand transfer activity enhancement. Adding LEDGF/p75 after 3′-processing gave a 2.5-fold strand transfer activity enhancement.

LEDGF/p75 was added before 3′-processing, strand transfer activity enhancement increased with increasing LEDGF/p75 dose and when the 3′-processing duration increased from 30 min to 90 min (Figures 3A and 3B). With a longer 3′-processing duration of 180 min, the strand transfer activity decreased relative to 90 min, possibly because of a gradual loss of activity from the integrase/donor DNA complex (Figure 3C). In contrast, when LEDGF/p75 was added after 3′-processing, no activity enhancement was observed. These results suggest that LEDGF/p75 played a role in the assembly of integrase on donor DNA and/or in the 3′-processing of the latter. Since the largest enhancement was observed at 0.15 μM LEDGF/p75 with 90 min of 3′-processing, these conditions were used in subsequent experiments.

LEDGF/p75 Increased the Assembly of Active Integrase/Donor DNA Complex and the Pseudo-First-Order Rate Constant of the Strand Transfer Reaction. The activity enhancement by LEDGF/p75 observed in Figure 3 could result from either an increased assembly of active integrase/donor DNA complex or an alteration of the complex that makes the strand transfer catalysis more efficient or both. To dissect the contribution of these two possible components, a strand transfer time course study was performed with a fixed 3′-processing/assembly duration of 90 min and three concentrations of LEDGF/p75 (0, 75, and 150 nM) (Figure 4, Table 2). Using unprocessed donor DNA, addition of 75 and 150 nM LEDGF/p75 before 3′-processing increased the active integrase/donor DNA complex formed, S_0, by 4- and 5.3-fold and the pseudo-first-order rate constant, k, of the strand transfer reaction by 2.9- and 3.7-fold respectively (Figures 4A, 4B, and 4C). In order to remove the necessity for 3′-processing, donor DNA with preprocessed 3′-end was also tested in the same experimental design. Addition of 75 and 150 nM LEDGF/p75 before integrase assembly on donor DNA increased S_0 by 1.6- and 3.4-fold and k by 1.6- and 2.7-fold respectively (Figures 4D, 4E, and 4F). These results suggest that LEDGF/p75 can increase the formation of active integrase/donor DNA complex whether or not 3′-processing is necessary and that the integrase/donor DNA complex formed in the presence of LEDGF/p75 is more efficient in catalyzing the strand transfer reaction as indicated by an increased rate constant. These experiments also showed that, in the absence of LEDGF/p75, the turnover rate of active integrase/donor DNA complex is less than 1 per hour (k = 0.68 h^{-1}).

The Integrase/Donor DNA Complex Formed in the Absence of LEDGF/p75 Becomes Refractory to the Enhancement Effect of LEDGF/p75. In order to determine in more detail whether LEDGF/p75 can have a stimulatory effect on strand transfer when added after the 3′-processing/assembly step, the time course experiment of Figure 4 was repeated to reflect this change in the time of addition of LEDGF/p75 while keeping all the other conditions identical (Figure 5 and Table 2). When 75 nM LEDGF/p75 was added at the start of the strand transfer step, it did not affect the strand transfer time course as compared to the control without LEDGF/p75, whether nonprocessed donor (Figure 5A and 5B) or 3′-processed donor (Figure 5D and 5E) was used. Both S_0 and k remained unchanged, indicating that neither the active integrase/donor DNA complex nor the efficiency of strand transfer was increased in contrast to what was observed when LEDGF/p75 was added before 3′-processing/assembly. This result suggests that the integrase/donor DNA complex formed in the absence of LEDGF/p75 becomes refractory to subsequent alteration by LEDGF/p75. Interestingly, when 150 nM LEDGF/p75 was added at the start of the strand transfer step, the strand transfer time course displayed a biphasic profile, discontinuous at the junction

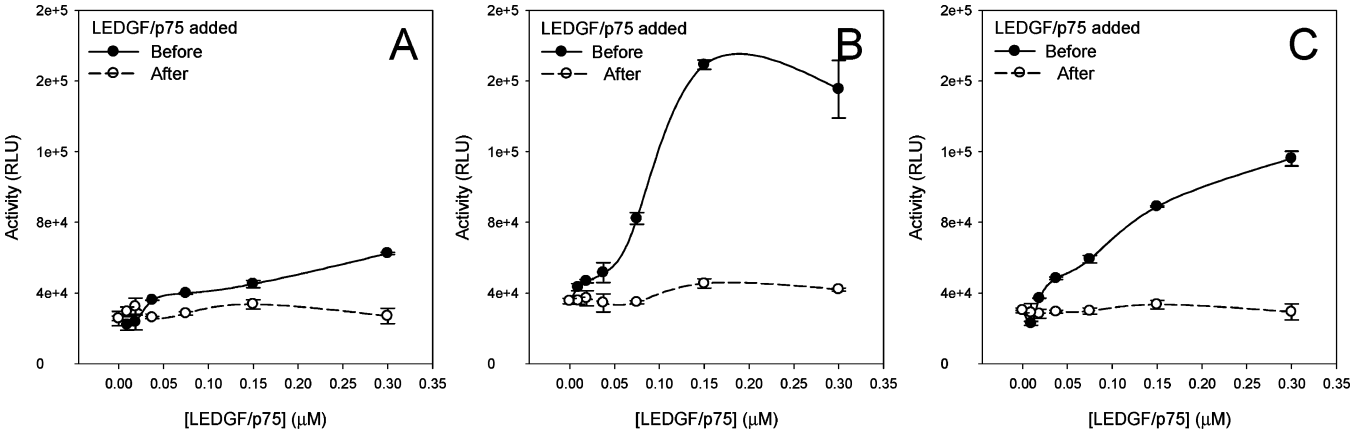


FIGURE 3: Effect of LEDGF/p75 on integrase assembly and 3′-processing. Standard strand transfer assay conditions were used with unprocessed donor DNA. Data points shown as mean of duplicate runs. The error bars represent the range of duplicate runs. Closed circles: LEDGF/p75 was added before 3′-processing. Open circles: LEDGF/p75 was added after 3′-processing. (A) 30 min 3′-processing. (B) 90 min 3′-processing. (C) 180 min 3′-processing.

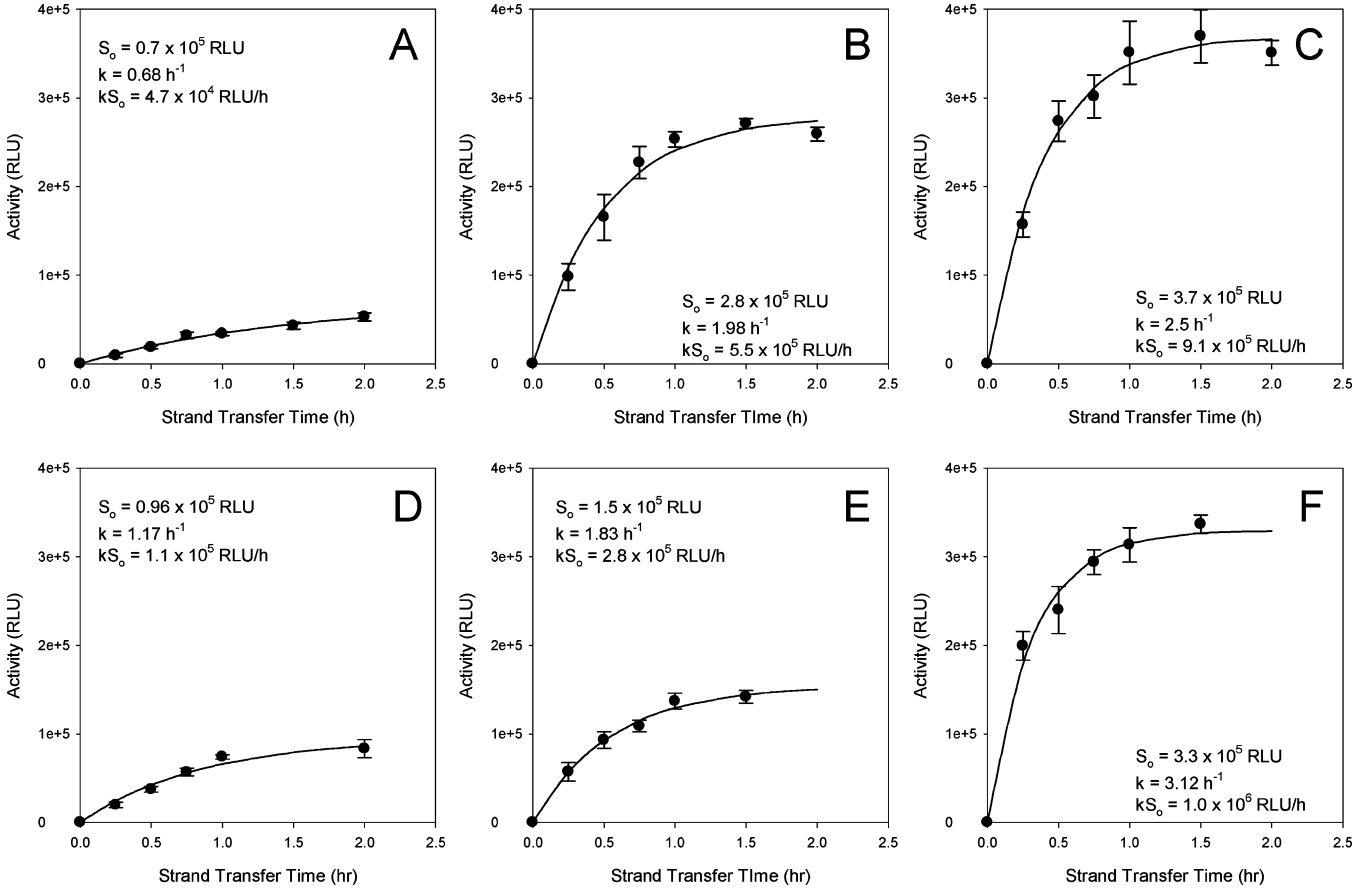


FIGURE 4: Effect of LEDGF/p75 added before assembly/3′-processing on the kinetics of strand transfer. Standard strand transfer assay conditions were used except that the assembly/3′-processing time was 90 min. Data points shown as mean of quadruplicate runs. The error bars represent the SD of quadruplicate runs. Unprocessed donor DNA was used in panels A, B, and C. 3′-processed donor DNA was used in panels D, E, and F. (A) and (D), no added LEDGF/p75; (B) and (E), [LEDGF/p75] = 75 nM; (C) and (F), [LEDGF/p75] = 150 nM.

Table 2: Effect of LEDGF/p75 on the Kinetic Parameters of Strand Transfer[a]

	LEDGF/p75 added before 3′-processing				LEDGF/p75 added after 3′-processing			
	unprocessed donor		3′-processed donor		unprocessed donor		3′-processed donor	
[LEDGF/p75] (nM)	S_0 (RLU)	k (h^{-1})	S_0 (RLU)	k (h^{-1})	S_0 (RLU)	k (h^{-1})	S_0 (RLU)	k (h^{-1})
0	0.7×10^5	0.68	0.9×10^5	1.17	1.12×10^5	1.03	0.60×10^5	1.64
75	2.8×10^5	1.98	1.5×10^5	1.83	1.15×10^5	0.88	0.60×10^5	1.77
150[b]	3.7×10^5	2.50	3.3×10^5	3.12	1.14×10^5	0.87	0.60×10^5	1.81
150[c]					1.41×10^5	0.86	1.01×10^5	1.40
fold-change[d]	5.3	3.7	3.7	2.7	2.3		2.7	

[a] This table summarizes the kinetic parameters determined in Figures 4 and 5. Assay conditions are described in the legends of Figures 4 and 5. S_0 and k were determined by fitting eq III or IV to the strand transfer time course. [b] First cohort of active integrase/donor DNA complex when LEDGF/p75 was added after 3′-processing. [c] Second cohort of active integrase/donor DNA complex when LEDGF/p75 was added after 3′-processing. [d] Fold-change between 150 nM and no LEDGF/p75. When two cohorts of integrase/donor DNA complexes were present, the sum of S_0 values from the two cohorts was used to calculate the fold-change.

of the two phases with a sudden increase in the rate (Figures 5C and 5F). Mathematically, a biphasic profile is indicative of the presence of two distinct phenomena. When the junction of the two phases is continuous, the two phenomena are occurring simultaneously throughout the time interval of observation. In contrast, when the junction of the two phases is discontinuous as in this case, the second phenomenon begins at the junction of the two phases from which point the two phenomena become simultaneous. With the benefit of this insight, we proceeded to curve fit the first phase (Figures 5C and 5F) using eq III to generate the solid line. This generated in the case of the unprocessed donor DNA values of $S_0 = 1.14 \times 10^5$ RLU and $k = 0.87$ h^{-1} almost identical to those of Figures 5A and 5B suggesting that the first phase represents the strand transfer time course catalyzed by the active integrase/donor DNA complex formed in the absence of LEDGF/p75. In order to separate the contribution of the second phenomenon from the first phenomenon in the second phase of the time course, the solid line was subtracted from the data points of the second phase (closed circles) to generate the open circles representing the time course of the second phenomenon. This time course can be analyzed by curve fitting using eq IV to generate the dashed line with values of $S_0 = 1.41 \times 10^5$ RLU, $k = 0.86$ h^{-1} and a time delay, $t_0 = 0.75$ h when nonprocessed donor DNA was used (Figure 5C). If the solid line (time course of the

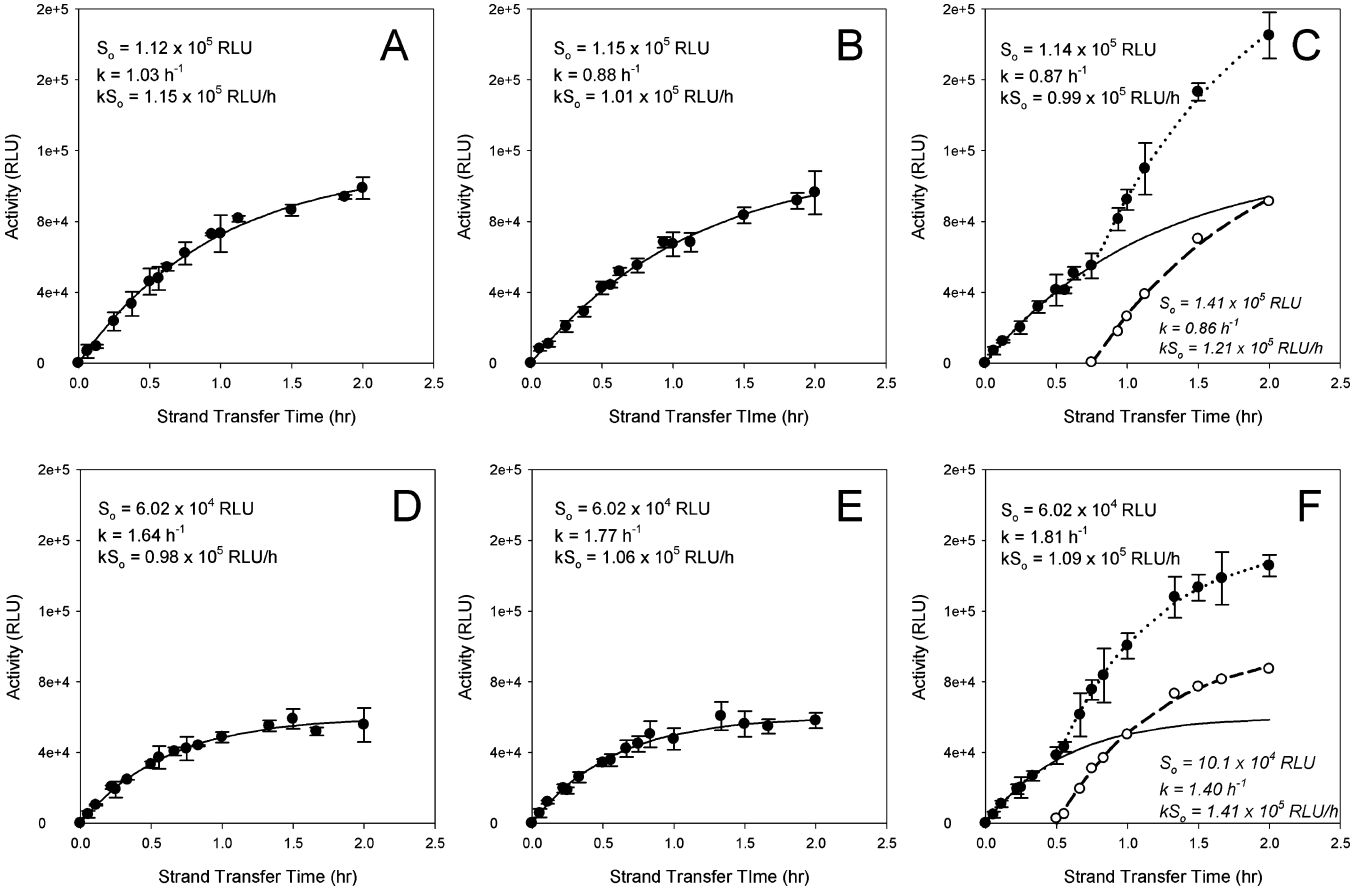


FIGURE 5: Effect of LEDGF/p75 added after assembly/3′-processing on the kinetics of strand transfer. Standard strand transfer assay conditions were used except that the assembly/3′-processing time was 90 min. Data points shown as mean of two independent experiments done each in quadruplicate. The error bars represent the SD of quadruplicate runs. Unprocessed donor DNA was used in panels A, B, and C. 3′-processed donor DNA was used in panels D, E, and F. (A) and (D), no added LEDGF/p75; (B) and (E), [LEDGF/p75] = 75 nM; (C) and (F), [LEDGF/p75] = 150 nM.

first phenomenon) is summed with the dashed line (time course of the second phenomenon), a dotted line fitting perfectly the original biphasic time course is generated. These results suggest that LEDGF/p75 at the higher concentration of 150 nM, when added at the start of the strand transfer step, is able to recruit the formation of a second cohort of active integrase/donor DNA complex by helping the free integrase to assemble onto and 3′-process the remaining free donor DNAs. The time delay of 45 min represents the time required for assembly/3′-processing before the second cohort of integrase/donor DNA becomes functional. The sum of S_0 values from the two cohorts is $(1.14 \times 10^5) + (1.41 \times 10^5) = 2.55 \times 10^5$ RLU, representing the total amount of integrase/donor DNA formed, and is 2.3-fold the S_0 value obtained in the absence of LEDGF/p75 (Figure 5C and Table 2). This 2.3-fold enhancement due to the recruitment of a second cohort is less pronounced than the 5.3-fold enhancement afforded by addition of 150 nM LEDGF/p75 before the 3′-processing step (Table 2). A similar recruitment of a second cohort of active integrase/donor DNA was also observed when 3′-processed donor DNA was used, except that the time delay of the second cohort was shortened from 45 min to 30 min (Figure 5F). This is consistent with the fact that 3′-processing is not required for preprocessed donor DNA and the shortened delay of 30 min represents the time required for assembly only.

LEDGF/p75 Decreases the K_m of Target DNA in the Strand Transfer Reaction. Since a decrease in the K_m of the strand transfer reaction could also potentially account for an increase in the IC_{50} of integrase inhibitors tested in the presence of LEDGF/p75 (Table 1), we measured the K_m of the strand transfer reaction in the absence and presence of 150 nM LEDGF/p75 by varying the concentrations of target DNA from 0 to 700 nM (Figure 6). The data was analyzed with eq V for the determination of K_m and V_{max}. Addition of 150 nM LEDGF/p75 before 3′-processing decreased the strand transfer K_m by 4.5-fold to 90 nM and increased V_{max} by 5-fold to 7.6×10^5 RLU/h (Figure 6). While an increase in V_{max} is consistent with an increase of active integrase/donor DNA complex seen in previous sections, a decrease in $K_m = (k_{-2} + k_{p2})/k_2$ has to reflect a decrease in the dissociation constant $K_d = k_{-2}/k_2$ of target DNA from integrase/donor DNA complex since we have already shown that $k_{p2} \sim k$ increased in the presence of LEDGF/p75 and could not have contributed to the decrease in K_m (Figure 4).

The Strand Transfer Enhancement Activity of LEDGF/p75 Is Dependent on Its Ability To Bind Integrase. To show that strand transfer enhancement activity of LEDGF/p75 seen in previous sections was dependent on its ability to bind integrase, we made an additional construct from pLEDGF75-6His by introducing the D366N mutation previously reported to disrupt the interaction of LEDGF/p75 with integrase (*8*). Both wild-type and mutant LEDGF/p75 were expressed and purified to >95% purity (Figure 7A) and assayed for strand transfer enhancement (Figure 7B). The wild-type LEDGF/p75 gave a maximum enhancement of ~16.2-fold. In

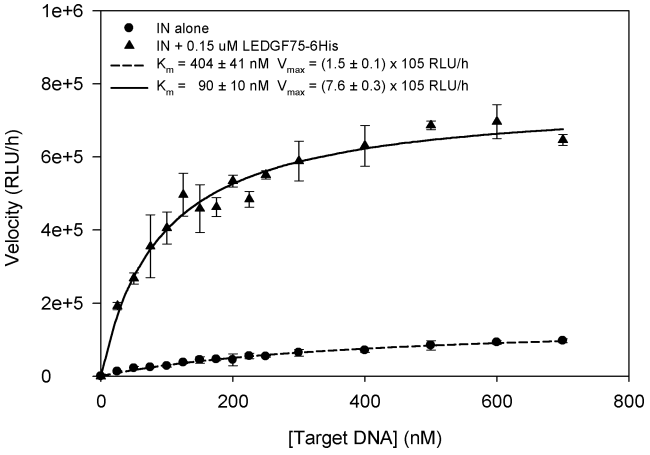


FIGURE 6: Effect of LEDGF/p75 on the velocity curve of strand transfer. Standard strand transfer assay conditions were used except that the 3′-processing time was 90 min. When present, LEDGF/p75 was added before 3′-processing. Data points are shown as mean of triplicate runs. The error bars represent the SD of triplicate runs. Circles: no added LEDGF/p75. Triangles: [LEDGF/p75] = 150 nM.

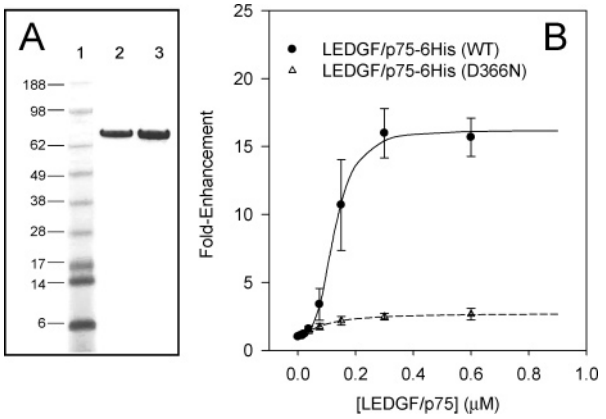


FIGURE 7: Comparison of wild-type and D366N LEDGF/p75. (A) Gel electrophoresis of purified recombinant LEDGF/p75: lane 2, LEDGF/p75-6His (wild-type); lane 3, LEDGF/p75 (D366N). (B) LEDGF/p75 dose response of strand transfer enhancement. Assay conditions are identical to those of Figure 2. Data points shown as mean of two independent experiments done in duplicate. The error bars represent the range of the two experiments. LEDGF/p75-6His (wild-type), EC_{50} = 125 nM, maximum enhancement = 16.2-fold; LEDGF/p75-6His (D366N), EC_{50} = 88 nM, maximum enhancement = 2.7-fold.

contrast, the D366N mutant with weakened interaction with integrase gave an enhancement that is diminished by 6-fold (i.e., ∼2.7-fold maximum enhancement) relative to the wild-type. We have also tested two mutant integrases (i.e., Q168A and L172A/K173A) previously reported to be incapable of binding to LEDGF/p75 against wild-type full-length LEDGF/p75. Wild-type full-length LEDGF/p75 did not significantly stimulate the activity of these two mutant integrases (see Supporting Information, Figure S2).

DISCUSSION

The integrase strand transfer assays used to date for the study of integrase activity enhancement by LEDGF/p75 relied on the detection of high molecular integration products on agarose gels or quantitative PCR of integration junctions which are cumbersome and not very amenable to the kinetic analysis of the enhancement effect. Using a 96-well plate based strand transfer assay with a chemiluminescent readout, we were able to obtain an estimate of the active integrase/donor DNA complex in our standard assay and study the kinetics of the strand transfer reaction to distinguish the effect of LEDGF/p75 on the enzyme/donor DNA complex formation from that on the rate constant of strand transfer catalysis and on the K_m for target DNA.

Under our standard strand transfer conditions, we estimated that <5 nM active integrase/donor DNA complex formed in the presence of 70 nM plate-bound donor DNA and 250 nM integrase, giving room potentially for >15-fold stimulation in integrase/donor DNA complex formation. Our analysis revealed that 150 nM LEDGF/p75, when added before the assembly/3′-processing step, was able to increase the formation of active integrase/donor DNA complex by ∼5-fold, the rate constant of strand transfer catalysis by ∼4-fold, and the affinity of the complex for target DNA by ∼4.5-fold. Both the increase in integrase/donor DNA complex and a decrease in K_m for target DNA are consistent with an observed increase in the IC_{50} for the competitive inhibitors of strand transfer in the presence of LEDGF/p75. The 4.5-fold decrease in K_m we observed in the enzymatic assay is also consistent with the previously reported 6.5-fold decrease in K_d for nonspecific target DNA at very similar concentrations of LEDGF/p75 (*21*) and can result from the ability of LEDGF/p75 to tether to chromosomal DNA through its AT-hook motif (*20*, *23*, *24*).

By exploring the effect of LEDGF/p75 addition after assembly/3′-processing, we discovered that the integrase/donor DNA complex formed in the absence of LEDGF/p75 is no longer susceptible to stimulation by subsequently added 75 nM LEDGF/p75. However, when LEDGF/p75 was added at a high enough concentration of 150 nM after the integrase/donor DNA complex formation, it was able to recruit the formation of a second cohort of integrase/donor DNA complex which became active for strand transfer after a delay of 45 min and contributed to a second phase in the strand transfer time course. This second phase did not result from the binding of LEDGF/p75 to the remaining integrase/donor DNA complex, as removal of free integrase by a wash after assembly/3′-processing made the second phase disappear, demonstrating that the second phase indeed resulted from the recruitment of a second cohort (data not shown).

This second cohort of complexes is responsible for the observed increase in the IC_{50} of integrase inhibitors when LEDGF/p75 was added after 3′-processing, since the strand transfer time was 60 min, 15 min longer than the required 45 min delay for the second cohort to become active (Table 1). In contrast, the effect of the second cohort cannot be seen in Figure 3 when LEDGF/p75 was added after 3′-processing because the strand transfer time was only 30 min, 15 min short of the 45 min required for the second cohort to become active. Similar results were obtained with 3′-processed donor DNA, except that the second cohort of integrase/donor DNA complex became active with a delay that is 15 min shorter than when unprocessed donor DNA was used. This suggests that 30 min is required for assembly of integrase onto donor DNA while an additional 15 min are required to fully process the 3′-end of donor DNA.

Our observations suggest that binding of LEDGF/p75 and donor DNA to integrase is sequential, with preferential binding of LEDGF/p75 first to integrase followed by an

enhanced binding of donor DNA to form a ternary complex with increased rate constant of catalysis for the strand transfer reaction and increased affinity for the target DNA. Our results also suggest that if donor DNA were allowed to bind first to integrase, LEDGF/p75 would not be able to bind subsequently to form the ternary complex or alternatively was still able to bind the integrase/DNA complex but would not be able to stimulate its activity. The recent cocrystal structure of the LEDGF/p75 IBD with the dimer of the integrase catalytic core domain (CCD) showed that the LEDGF/p75 IBD binds to a pocket at the CCD dimer interface (*28*). This LEDGF/p75 IBD binding site is shared by tetraphenylarsonium and its hydroxylated derivatives, a previously reported class of integrase inhibitors which displayed a 2- to 4-fold increase in IC_{50} if integrase is preassembled with donor DNA prior to addition of the inhibitors (*37*). There appears to be a parallel with the sequential binding of LEDGF/p75 before that of donor DNA we are proposing, in that the binding pocket for these compounds could become less accessible after the binding of donor DNA. To understand how LEDGF/p75 is able to increase integrase/donor DNA complex assembly and to verify this sequential binding model of LEDGF/p75 and donor DNA to integrase, a binding study using surface plasmon resonance spectroscopy can be performed to see (1) whether donor DNA binds to integrase/LEDGF/p75 complex with higher affinity than to integrase alone and (2) whether LEDGF/p75 can bind to integrase/donor DNA complex. If this sequential model is correct, the next question would be to determine how the assembly of donor DNA to integrase prevents the subsequent binding of LEDGF/p75. Is it by steric hindrance or allosteric hindrance? The key to this question may lie in the ability to obtain the crystal structure of the integrase/LEDGF/p75/donor DNA ternary complex.

SUPPORTING INFORMATION AVAILABLE

Integrase 3′-processing time course (Figure S1) and activity stimulation by LEDGF/p75 of integrase mutants defective for interaction with LEDGF/p75 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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