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The Interaction of LEDGF/p75 with Integrase Is Lentivirus-specific and Promotes DNA Binding*

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We have previously shown that the p75 isoform of the transcriptional co-activator lens epithelium-derived growth factor (LEDGF) interacts tightly with human immunodeficiency virus (HIV)-1 integrase (IN) and is essential for nuclear targeting of this protein in human cells (Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., De Clercq, E., and Debyser, Z. (2003) *J. Biol. Chem.* 278, 372–381; Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003) *J. Biol. Chem.* 278, 33528–33539). Here the interaction between recombinant LEDGF/p75 and HIV-1 IN was examined in a pull-down binding test. LEDGF/p75 was shown to increase the solubility of HIV-1 IN. Next, fluorescent correlation spectroscopy was used to measure the interaction of LEDGF/p75 or the complex of HIV-1 IN and LEDGF/p75 with a specific double-stranded DNA oligonucleotide. Whereas LEDGF/p75 displayed only a moderate affinity for DNA, it strongly promoted the binding of HIV-1 IN to DNA. This effect was specific for the p75 isoform of LEDGF and was not seen with p52. In the pull-down assay LEDGF/p75 interacted with HIV-1, HIV-2, and feline immunodeficiency virus IN, but not with the IN of human T-cell lymphotropic virus type 2, Moloney murine leukemia virus, or Rous sarcoma virus. These results strongly suggest that the interaction of LEDGF/p75 with IN is specific to lentiviridae. LEDGF/p75 stimulated the binding of HIV-1 and HIV-2 IN, but not Moloney murine leukemia virus or Rous sarcoma virus IN, to an aspecific DNA. These results provide supporting evidence for our hypothesis that LEDGF/p75 plays a role in the tethering of lentiviral IN to the chromosomal DNA.

Human immunodeficiency virus (HIV)[1]-1 belongs to the retroviridae that are characterized by reverse transcription of the diploid viral RNA genome into a double-stranded linear DNA molecule that is subsequently inserted into a host cell chromosome. Reverse transcription takes place in the cytoplasm of the infected cell and results in the formation of a compact and stable pre-integration complex (PIC) containing the viral reverse-transcribed genome and a number of virion-derived and cellular proteins. The family of the retroviridae can be broadly divided into simple and complex retroviridae, depending on their genomic composition and replication cycle. The retroviridae are then further subdivided into seven groups defined by evolutionary relatedness. Five of these groups represent retroviruses with oncogenic potential, and the other two groups are the lentiviruses and the spumaviruses (for a review, see Ref. 1). Lentiviridae (*e.g.* HIV-1, HIV-2, feline immunodeficiency virus (FIV), or simian immunodeficiency virus) are able to productively infect non-dividing cells, a feature that distinguishes them from oncoretroviridae (*e.g.* Moloney murine leukemia virus (Mo-MuLV), Rous sarcoma virus (RSV), and human T-cell lymphotropic virus (HTLV)-2), which require cell division for productive infection (2, 3). The viral integrase (IN) catalyzes the integration of the viral cDNA into the host genomic DNA, a process that is essential for replication and results in a provirus that will remain present as long as the cell survives (for reviews, see Refs. 4–6).

HIV-1 IN is a 32-kDa protein that consists of three distinct structural domains (7): the N-terminal zinc-binding domain required for oligomerization (8–10), the central catalytic core, and the less highly conserved C-terminal domain thought to be involved in DNA binding (11) and oligomerization of IN *in vitro* (12). The functional holoprotein required for concerted integration of two long terminal repeat ends is believed to exist as a homodimer of two tetramers (13, 14). In cells stably overexpressing IN, the enzyme remains stably associated with condensed chromosomes during mitosis (15, 16).

The insight has grown that HIV relies on cellular proteins for completion of its replication cycle. Identification and characterization of these cellular cofactors will increase our understanding of the viral replication cycle and aid in the development of new antiviral drugs. Various cofactors of the lentiviral integration process have been proposed. Integrase interactor 1, a component of the SWI/SNF chromatin remodeling complex (17–19), interacts directly with HIV-1 IN and stimulates the integration

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[1] The abbreviations used are: HIV, human immunodeficiency virus; FCS, fluorescence correlation spectroscopy; FIV, feline immunodeficiency virus; HTLV, human T-cell lymphotropic virus; IN, integrase; LEDGF, lens epithelium-derived growth factor; Mo-MuLV, Moloney murine leukemia virus; PIC, pre-integration complex; RSV, Rous sarcoma virus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Ni-NTA, nickel-nitrilotriacetic acid.

reaction *in vitro* (20, 21). Other cellular host factors might participate in HIV integration. The high mobility group protein HMG-1a, a non-histone chromosomal protein involved in transcriptional control and nuclear architecture, was identified as a cellular host factor essential for PIC activity *in vitro* (22). Human EED, a cellular protein interacting with HIV-1 matrix protein and acting as a transcriptional repressor and gene silencer, was found to interact with HIV-1 IN and to stimulate the integration reaction *in vitro* (23). Other cellular factors, such as the barrier to autointegration factor and Ku, have been shown to co-purify with PICs. Barrier to autointegration factor is thought to prevent intramolecular integration (24). The exact function of all these factors during *in vivo* integration remains to be determined.

Recently, we have identified the p75 isoform of lens epithelium-derived growth factor (LEDGF/p75) as a tight binding partner of HIV-1 IN in human cells (13). LEDGF/p75 was shown to enhance the enzymatic activity of recombinant HIV-1 IN. Expression of LEDGF/p75 is required for the association of IN with mitotic chromosomes, suggesting a role in tethering IN to DNA (16). Although the exact cellular function of LEDGF/p75 is still unknown, it has been suggested to play a role in transcriptional regulation because it is isolated from HeLa cell extracts as an interactor of the transcriptional co-activator PC4 (25). LEDGF/p75 is a member of the hepatoma-derived growth factor family and has been proposed to play a protective role during stress-induced apoptosis (26–28). DNA binding of LEDGF/p75, with specificity for stress response DNA elements, has been reported (29). A second protein product, p52, is generated from the LEDGF/p75 gene as a result of alternative splicing of the pre-mRNA (25, 30). Although p52 was found to be a more general and stronger transcriptional co-activator *in vitro* than LEDGF/p75 (31), it does not interact with HIV-1 IN *in vitro* or in living cells (16).

In this report, we used fluorescence correlation spectroscopy (FCS) to study the effect of LEDGF/p75 on the binding of IN to different DNA substrates. LEDGF/p75, but not p52, clearly stimulated the binding of HIV-1 IN to DNA. Moreover, in both pull-down and FCS assays, the interaction of LEDGF/p75 with IN proved to be specific for lentiviridae.

EXPERIMENTAL PROCEDURES

Plasmids for Bacterial Expression—The constructs pCPnat75 and pKBnat52 were used for the bacterial expression of non-tagged LEDGF/p75 and p52 proteins as described previously (16). The plasmid pKBIN6H was used for the bacterial expression of the C-terminal His_6-tagged form of HIV-1 IN (16), and the N-terminal-tagged form of HIV-1 IN was expressed from pRP1012 (32). DNA fragments containing the IN open reading frame of HIV-2 (33), FIV (34), and RSV (35) were cloned into pET-20b(+) vector (Novagen, VWR, Leuven, Belgium) as fusions to a C-terminal His_6 tag, using the NdeI and SalI sites. The fragments were PCR-amplified using the following primers and templates: 5′-CGCGTCGACTGCCATTTCTCCATCCT, 5′-CGCCATATGTTCCTGGAAAAAATAGAGC, and pRP1013 to obtain pKBHIV2IN; 5′-CGCGTCGACCTCATCCCCTTCAGGAAGAGC, 5′-CGCCATATGTGGGTTGACAGAATTGAGGAAG, and pRP825 to obtain pKBFIVIN; and T7 promoter primer and 5′-CGCGTCGACTGCAAAAAGAGGGCTCGCCTC to obtain pKBRSVIN. HTLV-2 IN with a C-terminal His_6 tag was expressed from the plasmid pHTLV2 (36), and for bacterial expression of Mo-MuLV IN, the plasmid pETINH1 was used (37). All plasmid constructs used in this work were verified via sequence analysis.

Production and Purification of Recombinant Proteins—Non-tagged LEDGF/p75 and p52 proteins were produced from the plasmids pCPnat75 and pKBnat52, respectively, in *Escherichia coli* B BL21(DE3) and purified as described previously (16). The purification of HIV-2 IN, FIV IN, and RSV IN was done as described for HIV-1 IN (16). HTLV-2 IN was first enriched by batch purification on Ni-NTA-agarose (Qiagen) and then manually loaded on a 1-ml HiTrap™ Heparin column (Amersham Biosciences) and eluted with 1 M NaCl, 7.5 mM CHAPS, and 50 mM Tris (pH 7.2). The fractions were analyzed by SDS-PAGE, and the peak fractions were supplemented with 5 mM dithiothreitol plus 10% glycerol and frozen at −80 °C. The purification of Mo-MuLV IN was performed as described previously (38). The purified recombinant proteins were concentrated by ultrafiltration using Centricon10 (Millipore) or Vivaspin 15R (Vivascience, Hannover, Germany). All IN protein concentrations were measured using the Bradford assay (Bio-Rad); the proteins were separated by 11% SDS-PAGE and visualized with Coomassie Blue R-250.

His_6 Tag Integrase Pull-down Assay—Binding of IN to LEDGF/p75 was assayed in 25 mM Tris-HCl (pH 7.4), 0.1% Nonidet P-40, and 20 mM imidazole containing 150 or 400 mM NaCl, in the presence of 1 mM $MgCl_2$ (binding buffer). 1 μg of recombinant His_6-tagged IN was incubated with 1–3 μg of LEDGF/p75 in 200 μl of binding buffer supplemented with 1 mg/ml bovine serum albumin. Following a 30-min incubation at 4 °C, the mixtures were centrifuged for 2 min at 13,000 rpm at 4 °C to remove aggregated protein. The supernatant was supplemented with 40 μl of Ni-NTA-agarose and stirred for an additional 30 min. The agarose beads were recovered by centrifugation for 2 min at 13,000 rpm at 4 °C and washed with 500 μl of binding buffer. Bound proteins were eluted in 40 μl of binding buffer supplemented with 200 mM imidazole and 1% SDS and analyzed by 11% SDS-PAGE followed by staining with Coomassie Blue R-250 (16).

For the solubility assay, the same amounts of IN and bovine serum albumin were added to 200 μl of binding buffer with a NaCl concentration ranging between 100 and 500 mM in the absence or presence of LEDGF/p75. After a 30-min incubation at 4 °C, the mixtures were centrifuged for 2 min at 13,000 rpm at 4 °C to remove aggregated proteins. The supernatant was precipitated with trichloroacetic acid by adding 1 volume of 50% trichloroacetic acid to the protein sample. Following a 30-min incubation on ice, the samples were centrifuged for 15 min at 13,000 rpm at 4 °C. The pellets were washed with cold acetone and centrifuged again to remove the supernatant. The pellet was dried and redissolved in SDS-PAGE loading buffer for analysis on SDS-PAGE.

FCS Instrumentation—A commercial FCS set up (the ConfoCor I of Zeiss) was used as described previously (39, 40). In this configuration, an objective lens of type C-Apochromat 40x/1.2W was used, the pinhole diameter was 45 μm in all experiments, and a typical size of the excitation volume was 1.4 fl. The 543 nm line of the HeNe ion laser and the 488 nm line of an Argon ion laser were applied in all experiments, attenuated with a 0.3 optical density neutral density filter for the DNA binding experiments. The laser beam was focused at about 180 μm above the bottom of the Nunc cuvettes (Nalge Nunc International, Naperville, IL) in a typical volume of 10 μl. The data electronics and software (Borland Delphi) were used as described previously (41).

DNA Binding Assay—The DNA binding assay was based on fluorescence fluctuations analysis (11). This was carried out on the one hand with synthetic oligonucleotides that resemble the U5 long terminal repeat ends of the genome of each specific retrovirus analyzed and on the other hand with one aspecific DNA substrate. For each DNA substrate, one oligonucleotide was fluorescence-labeled with a tetramethylrhodamine derivative (TAMRA; Molecular Probes), at the 5′ end. For HIV-1, we used INT1-TMR5 (5′-TGTGGAAAATCTCTAGCAGT) and INT2 (5′-ACTGCTAGAGATTTTCCACA); for RSV, we used RSVINT1-TMR5 (5′-ATTGCATAAGACTACATT) and RSVINT2 (5′-AATGTAGTCTTATGCAAT); for Mo-MuLV, we used Mo-MuLVINT1-TMR5 (5′-GTCAGCGGGGGTCTTTCATT) and Mo-MuLVINT2 (5′-AATGAAAGACCCCCGCTGAC); and for HIV-2, we used HIV2INT1-TMR5 (5′-GCAGGAAAATCCCTAGCAGG) and HIV2INT2 (5′-CCTGCTAGGGATTTTCCTGC). For the aspecific DNA substrate 5′-CCTGCTAGGGATTTTCCTGC, TCTTCTTCTTCTTCTTCTGTGCACTCTTCTTCTTCT and GCGCACGCGTGTGCACACTCGAGTACGTACATGCATGC were ordered and labeled at the 5′ end with BODIPY. All oligonucleotides were synthesized and purified by Proligo (Paris, France). We purified the oligonucleotides further by gel electrophoresis in a denaturing urea gel (42). For substrate preparation, equimolar amounts of complementary oligonucleotides were annealed in 20 mM HEPES (pH 7.5) containing 100 mM NaCl. The samples were incubated at 80 °C for 1 min and cooled to 20 °C over the course of ~90 min. The final DNA concentration of the fluorescent double-stranded DNA was determined using ConfoCor I. The effect of LEDGF/p75 on the DNA binding properties of IN was tested in the reaction buffer normally used for enzymatic assays. This buffer contained the following final concentrations: 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10% dimethyl sulfoxide, and 5% polyethylene glycol 8000 and will be referred to henceforth as the reaction buffer. The DNA substrate concentration was kept constant at 30 nM, whereas the IN concentration varied. After an incubation of the samples for 10 min at room temperature, the measurements were performed during a 60-s period. Each sample was

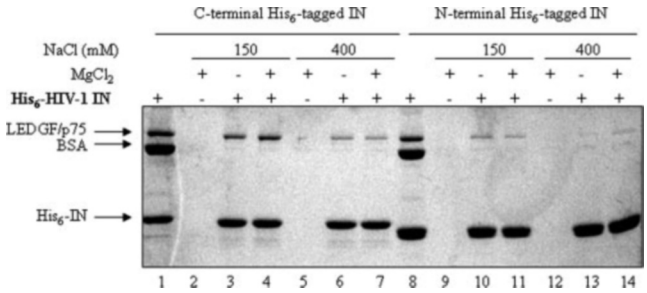

FIG. 1. **A His_6 tag at the N terminus of HIV-1 IN reduces interaction with LEDGF/p75.** Recombinant LEDGF/p75 was incubated with HIV-1 IN containing a His_6 tag at the N (*lanes 10*, *11*, *13*, and *14*) or C terminus (*lanes 3*, *4*, *6*, and *7*) in the salt and $MgCl_2$ concentrations indicated. The complexes were bound on a Ni^{2+} chelating resin. *Lanes 1* and *8* reflect the protein input in the reactions: bovine serum albumin (*BSA*), LEDGF/p75, and His_6-IN were loaded in the same amounts present in the binding reactions. The gel was stained using Coomassie Blue R-250. There is less LEDGF/p75 detectable in *lanes 10*, *11*, *13*, and *14*, where N-terminally His-tagged IN was used, compared with *lanes 3*, *4*, *6*, and *7*, where C-terminally His-tagged IN was used. Addition of $MgCl_2$ increased the binding of LEDGF/p75 to IN (compare *lane 4* with *lane 3*).

measured 10 times. The data were subsequently analyzed as described previously, using the quantile plot analysis method (12).

RESULTS

Interaction of HIV-1 Integrase with LEDGF/p75 as Analyzed by a Pull-down Binding Assay—To study the interaction between IN and LEDGF/p75 *in vitro* we optimized a specific binding assay, based on the ability of Ni-NTA-agarose to bind His_6-tagged proteins (16). We have previously demonstrated that LEDGF/p75 but not p52 specifically interacts with HIV-1 IN in this test (16). After pre-incubation of His_6-tagged HIV-1 IN with recombinant LEDGF/p75, HIV-1 IN is bound to Ni-NTA-agarose, washed, and eluted with imidazole. Recombinant LEDGF/p75 is readily recovered in a stable complex with IN. Finally, the proteins in the complex are identified in a SDS-PAGE gel stained with Coomassie Blue R-250.

First we examined the effect of a N- *versus* C-terminal His_6 tag on the interaction between LEDGF/p75 and HIV-1 IN as well as the effect of the NaCl concentration in the buffer. Heretofore we cloned the His_6 tag at the C terminus or the N terminus of HIV-1 IN. In comparison with the protein with the His_6 tag at the C terminus, N-terminal-tagged HIV-1 IN pulled down less LEDGF/p75 (Fig. 1). This result is in agreement with our previous findings that the N-terminal domain of IN enhances the affinity of IN for LEDGF/p75 (16). Therefore, all further experiments were carried out with IN tagged at the C terminus.

The binding assays were performed in both 150 and 400 mM NaCl. Although the interaction of IN with LEDGF/p75 is strongest in 150 mM NaCl (Fig. 1, compare the band for LEDGF/p75 in *lanes 3* and *4* with *lanes 6* and *7* and in *lanes 10* and *11* with *lanes 13* and *14*), the precipitation of IN is also more pronounced in the presence of 150 mM NaCl (Fig. 2). This effect was minimal in the experiment shown in Fig. 1, but it was more pronounced with the other retroviral INs used in the present study. Therefore, the following binding experiments were always performed in both 150 and 400 mM NaCl.

LEDGF/p75 Enhances the Solubility of HIV-1 IN—To study the effect of LEDGF/p75 on the solubility of HIV-1 IN, we performed a precipitation experiment to determine the NaCl concentration required to keep IN in solution. We used the buffer of the pull-down assay, with NaCl concentrations ranging between 100 and 500 mM (Fig. 2). After 30 min of incubation, the samples were centrifuged, and the pellets were separated from the supernatant, which was precipitated with trichloroacetic acid. All samples were redissolved and run in a SDS-PAGE gel. In the absence of LEDGF/p75, IN precipitated in salt conditions of ≤350 mM. In the presence of LEDGF/p75, however, IN remained soluble at a NaCl concentration above 200 mM. Addition of bovine serum albumin did not increase the solubility of IN, indicating that the increase in solubility of IN by LEDGF/p75 is a specific effect.

LEDGF/p75 Enhances the Binding of HIV-1 IN to DNA—We have previously established an FCS-based assay to analyze the binding of HIV-1 IN to a fluorescence-labeled 20-mer double-stranded DNA resembling the HIV-1 U5 long terminal repeat sequence (12). In this study at a final NaCl concentration of 50 mM, the dissociation constant (K_d) for the IN·DNA complex was 208 ± 26 nM. We then investigated the effect of the addition of recombinant LEDGF/p75 on the formation of the IN·DNA complex. The K_d for this LEDGF-DNA complex was estimated at 1110 ± 86 nM. Next, increasing concentrations of LEDGF/p75 were added to the DNA at various concentrations of HIV-1 IN. By plotting the calculated amounts of free proteins and complexes bound to the DNA, we could demonstrate a clear stimulation by LEDGF/p75 of the binding of HIV-1 IN to DNA (Fig. 3). From the slope of the binding plot at low IN concentrations, the dissociation constant for the IN·LEDGF/p75·DNA complex was calculated at 6.17 ± 0.88 nM, corresponding to a >33-fold increase in binding. We also investigated the binding of the p52 isoform to DNA. This protein bound to DNA with a higher affinity than LEDGF/p75. The calculated apparent K_d was 763 ± 303 nM. Upon addition of p52, no stimulation of the binding of IN to DNA was seen (Fig. 3). Overall binding merely represented the additive effect of the binding of each protein to the DNA (data point at IN = 0 concentration).

The Interaction between LEDGF/p75 and IN Is Lentivirus-specific—The specificity of binding of various retroviral INs to human LEDGF/p75 was tested in the pull-down binding assay. The INs of the following lentiviridae were included in the experiment: HIV-1, HIV-2, and FIV, as well as the INs of the retroviridae HTLV-2, Mo-MuLV, and RSV. All C-terminal His_6-tagged INs were expressed in bacteria and purified by Ni-NTA and Heparin chromatography. The purification scheme is described under "Experimental Procedures" and was identical for all INs, except for Mo-MuLV and HTLV-2 IN. Of note, HTLV-2 IN displayed a very low solubility and precipitated even in 1 M NaCl, but LEDGF/p75 did not enhance its solubility (data not shown). The purity of the different IN preparations was verified with SDS-PAGE, and the proteins were used at equimolar concentrations in the pull-down binding assay.

The results of these interaction studies are shown in Fig. 4. The IN of HIV-1, HIV-2, and FIV showed a strong and specific interaction with human LEDGF/p75 (Fig. 4*A*). No interaction was detected with the IN of human HTLV-2, murine Mo-MuLV, or avian RSV (*lanes 4* and *6* on all gels in Fig. 4*B*) because no LEDGF/p75 was pulled-down for these INs. This lack of interaction with retroviral IN was confirmed by a more sensitive detection of LEDGF/p75 by Western blotting using a monoclonal anti-LEDGF p75/p52 antibody (data not shown). The results demonstrate that the binding of LEDGF/p75 to IN is lentivirus-specific. In a parallel yeast two-hybrid experiment, interaction was shown between LEDGF/p75 and the integrases of HIV-1 and simian immunodeficiency virus from rhesus macaques (SIVmac) (data not shown).

Stimulation of the Binding of IN to DNA by LEDGF/p75 Is Lentivirus-specific—Next, lentiviral specificity of the functional interaction between IN and LEDGF/p75 was examined in the FCS assay (Fig. 5). To model tethering of IN to chromosomal DNA, we first used a single aspecific double-stranded

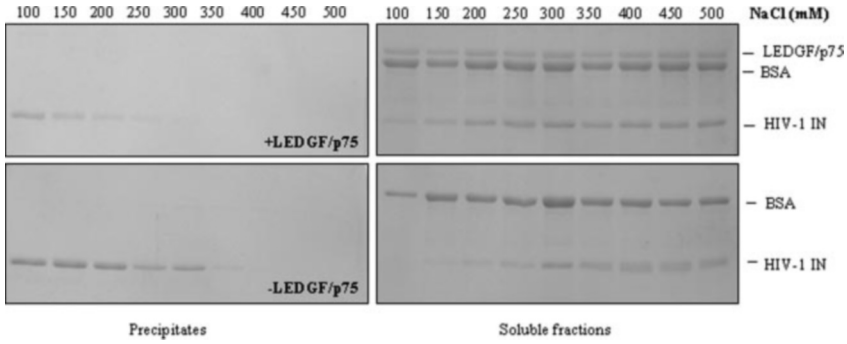

FIG. 2. **LEDGF/p75 enhances the solubility of HIV-1 IN.** HIV-1 IN was added to the binding buffer of the pull-down assay in a variety of salt concentrations ranging from 100 to 500 mM NaCl. LEDGF/p75 was added in the experiment shown in the *top panels*. After 30 min of incubation, the samples were centrifuged, and the pellets were separated from the supernatant, which was precipitated with trichloroacetic acid. All samples were redissolved and separated by SDS-PAGE. Without LEDGF/p75, HIV-1 IN precipitated in buffers containing <350 mM salt, whereas the addition of LEDGF/p75 increased the solubility of IN.

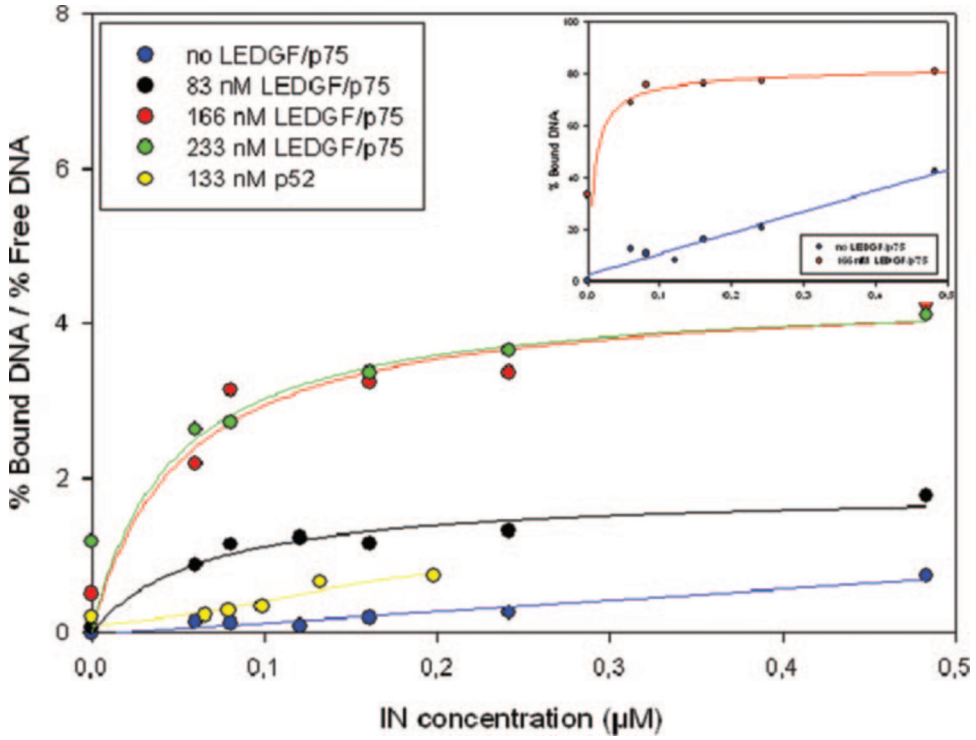

FIG. 3. **Stimulation of the DNA binding of IN by addition of LEDGF/p75.** A fluorescence-labeled 20-mer specific double-stranded DNA was used as substrate for evaluating the binding of HIV-1 IN and the effect of the addition of LEDGF/p75. The amounts of free and bound DNA were calculated using FCS and spike analysis (12). This ratio was plotted against the initial IN concentration. Different concentrations of LEDGF/p75 were added, and the binding affinity of the IN·LEDGF/p75 complex formed was measured. An apparent binding constant was calculated from the slope of the curve at low IN concentrations (K_d = 208 ± 26 nM for IN and 6.17 ± 0.88 nM for IN·LEDGF·DNA). Each data point represents the average of 10 measurements. In the *inset*, the percentage of bound DNA is plotted against IN concentration.

DNA for the different integrases tested. The final NaCl concentration in this assay was 130 mM. Whereas LEDGF/p75 stimulated the binding of both HIV-1 IN and HIV-2 IN to DNA (Fig. 5*A*), no stimulation of the binding of RSV IN or Mo-MuLV IN to the DNA was detected, even when LEDGF/p75 was added at a concentration of 166 nM (Fig. 5*B*). The respective dissociation constants are given in Table I. To compare stimulation of IN binding to specific DNA, each recombinant integrase was subsequently tested in the presence of its cognate oligonucleotide DNA mimicking the 5′ long terminal repeat end (Fig. 5, *C* and *D*). Again, DNA binding of lentiviral but not retroviral integrases was stimulated upon addition of LEDGF/p75.

DISCUSSION

The present data provide further evidence that LEDGF/p75 acts as a cellular cofactor for lentiviral integration. We proved the lentiviral specificity of the IN-LEDGF/p75 interaction by demonstrating that LEDGF/p75 interacts with the lentiviral INs of HIV-1, HIV-2, SIVmac, and FIV but not with the retroviral INs of HTLV-2, RSV, or Mo-MuLV. Furthermore, we shed light on the potential role of LEDGF/p75 during HIV replication by demonstrating a direct stimulation of the binding of lentiviral INs to DNA by LEDGF/p75, but not by p52. The molecular mechanism of this cofactor is apparently based on the stimulation of the binding of IN to the DNA. Our findings support our hypothesis that LEDGF/p75 plays a role in tethering IN to the chromosomal DNA (12, 15).

We have unambiguously demonstrated the specificity of interaction of LEDGF/p75 with lentiviral integrases both in a pull-down interaction assay and with FCS. While this article was in preparation, Llano *et al.* (43) reported as well on the role of LEDGF/p75 in the nuclear accumulation of HIV-1 and FIV but not Mo-MuLV IN.

In the FCS experiments, we first compared the DNA binding of the different INs by using the same nonspecific DNA substrate, thus modeling binding to the chromosomal DNA. Stimulation of binding to the DNA by LEDGF/p75 was observed for HIV-1 and HIV-2 IN but not for RSV or Mo-MuLV IN. When using specific DNA substrates, the same lentiviral specificity of LEDGF/p75 was observed.

Stimulation by LEDGF/p75 was more pronounced for HIV-1

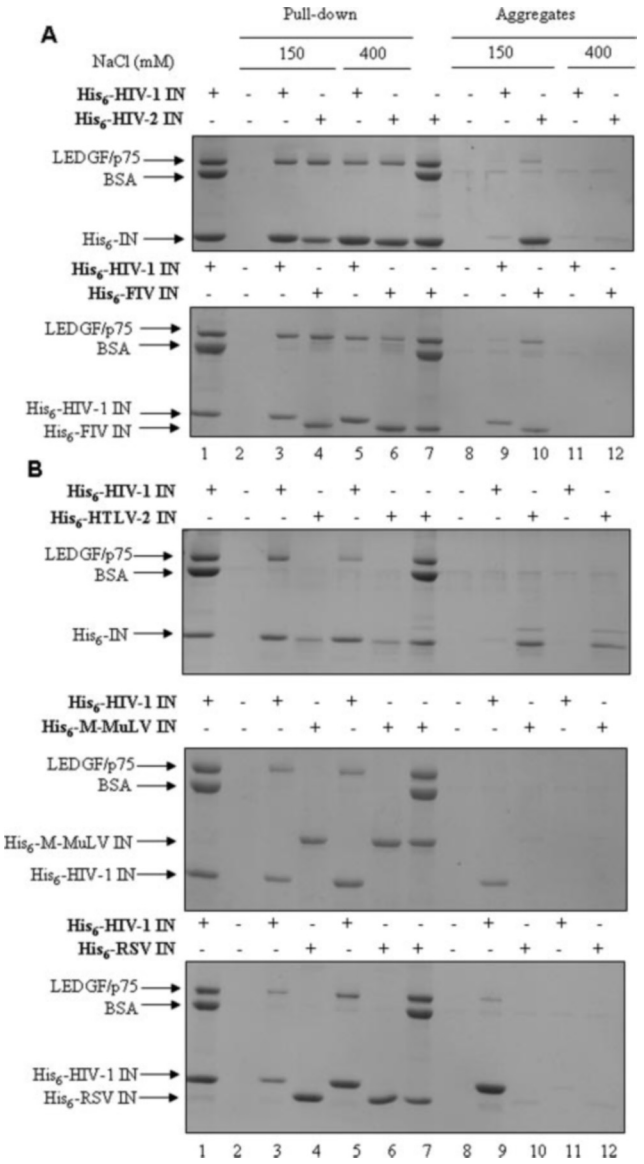

FIG. 4. **The interaction between LEDGF/p75 and IN is specific for lentiviridae.** The pull-down assay was performed for the INs from the following retroviruses: HIV-2, FIV, HTLV-2, Mo-MuLV, and RSV. HIV-1 IN was used as a control sample. The salt concentration is indicated *above* the gel; in all reactions, 1 mM $MgCl_2$ was added. Recombinant LEDGF/p75 was incubated with His_6-tagged HIV-1 IN, and the complexes were recovered on Ni^{2+} chelating agarose. Part of the proteins aggregated during the test (see *lanes 8–12* in all gels). The respective positions of the proteins are indicated to the *left* of the gel; the gel was stained using Coomassie Blue R-250. *Lane 1* always reflects the protein input for the control reactions. *Lane 7* reflects the protein input for the pull-down with non-HIV-1 IN. *A*, for both HIV-2 IN and FIV IN, LEDGF/p75 can be readily detected after pull-down (*lanes 4* and *6* in the two gels). *B*, HTLV-2, Mo-MuLV, and RSV IN do not directly interact with LEDGF/p75 because no LEDGF/p75 co-precipitated in the pull-down assay (*lanes 4* and *6* in the three gels).

than HIV-2 IN with both specific and aspecific DNA. The stimulation was also more pronounced with specific than aspecific DNA, although LEDGF/p75 by itself showed more affinity for the aspecific DNA substrate. Based on these data, we cannot rule out the possibility that LEDGF/p75 is also involved in tethering IN to the viral DNA ends. The evidence for the presence of LEDGF/p75 in the PIC (43) is in agreement with this hypothesis.

Interestingly, we observed a clear increase in the solubility of recombinant HIV-1 IN complexed with LEDGF/p75. This points to a potential strategy for crystallization of this holoprotein complex. The increased affinity of IN for DNA may facilitate co-crystallization of IN with a DNA substrate.

The specific interaction of LEDGF/p75 with lentiviral but not retroviral INs raises the question of whether retroviruses have a different mechanism of integration and/or interact with other host proteins. The human origin of the LEDGF/p75 used cannot be the culprit because interaction was found with FIV IN as well. Lentiviruses differ foremost from the other retroviruses in their ability to infect non-dividing cells (2, 3). This has resulted in a search for the possible import factors of the lentiviral pre-integration complex. Although the mechanism underlying the nuclear import of the lentiviral PIC has not been clarified, viral proteins such as Vpr, matrix protein, and IN as well as the central DNA flap have been implicated (44–49). Recent data by Maertens *et al.* (50) demonstrate that LEDGF/p75 contains a functional nuclear localization signal, but no role in the nuclear import of IN was detected. Devroe *et al.* (51) have previously shown that by fusing IN to a nuclear export signal, the nuclear localization of constitutively expressed HIV-1 IN was not abolished. They suggested that HIV-1 IN is trapped in the nucleus, possibly through interaction with chromatin or direct binding to DNA. We have previously reported that LEDGF/p75 is necessary for the nuclear localization of HIV-1 IN (16). By gene silencing of LEDGF/p75, the nuclear accumulation of IN was abolished. The recent data of Llano *et al.* (43) confirm our observations; a direct role of LEDGF/p75 in nuclear import was questioned, but a putative role in chromosomal targeting was put forward. Finally, our FCS analysis supports the view that LEDGF/p75 tethers IN to the chromosome by providing direct evidence for increased binding of HIV-1 IN to DNA in the presence of this cellular cofactor. Together, these findings point to LEDGF/p75 as the nuclear and chromosomal trap of IN rather than as a nuclear import factor for the PIC, although an additional role of LEDGF/p75 in nuclear import cannot be excluded at this moment.

Besides their different ability to infect cells, another important difference between lentiviral and retroviral integration is the selection of integration sites (for a recent review, see Ref. 52). Replication of retroviruses and retrotransposons depends on the selection of a favorable site for integration in the chromosome. Integration is known to occur in a non-sequence-specific manner, so many chromosomal sites can host integration. A recent study by Wu *et al.* (53) compared integration targeting in the human genome by HIV and Mo-MuLV vectors. For HIV, integration is favored in transcriptional units. Comparison with transcriptional profiling data supports the idea that active genes are preferred. Mo-MuLV integration preferentially occurs near the start of transcriptional units. This study has been confirmed and supplemented with data for avian sarcoma/leukosis virus by Mitchell *et al.* (54), showing that avian sarcoma/leukosis virus displays only weak preference for active genes and no preference for transcriptional start regions. Whereas the detailed mechanism of retroviral integration targeting is unknown, these studies can be easily accommodated in tethering models (for review, see Ref. 52). In one version of such a model, the binding of the PIC of Mo-MuLV to transcription factors or modified histones bound at or near the 5′ end of genes promotes local integration. HIV might similarly interact with positive factors bound within transcription units (53). Interestingly, it has been shown that LEDGF/p75 attaches to the chromatin during the G_2 phase of the cell cycle (55). We hypothesize that the temporal attachment of LEDGF/p75 to the chromatin targets the integration of HIV-1 proviral DNA to specific genomic sites of actively transcribed genes (56). Moreover, LEDGF/p75 was first identified as an interacting

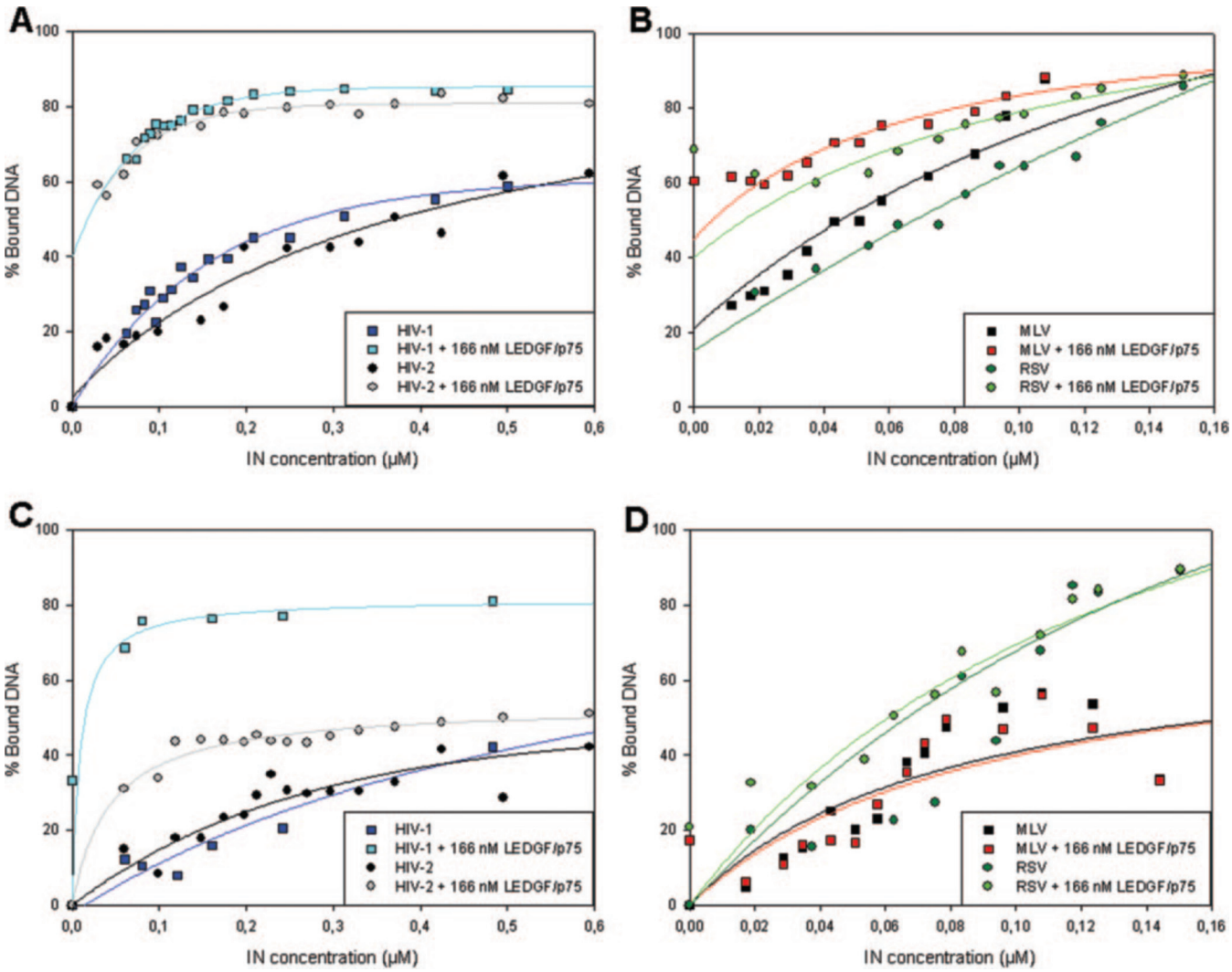

FIG. 5. **The stimulation of DNA binding of IN by the addition of LEDGF/p75 is specific for lentiviridae.** A fluorescence-labeled 20-mer double-stranded DNA was used as substrate to evaluate the binding of different INs and the effect of the addition of LEDGF/p75. The amounts of free and bound DNA were calculated using FCS, and the percentage of bound DNA was plotted against the initial IN concentration. LEDGF/p75 was added at a concentration of 166 nM. *A*, stimulation of the DNA binding of HIV-1 and HIV-2 IN by addition of LEDGF/p75 and using an aspecific DNA substrate. *B*, no stimulation of DNA binding of Mo-MuLV or RSV IN when using an aspecific DNA substrate. *C*, stimulation of the DNA binding by LEDGF/p75 when using specific DNA substrates for HIV-1 and HIV-2 IN. *D*, specific substrates for Mo-MuLV and RSV IN were used. Every data point represents the average of 10 measurements.

TABLE I
Dissociation constants (K_d) for IN·DNA binding with or without LEDGF/p75

The constants were calculated from the slopes of the binding plots at low IN concentrations (Fig. 5). The FCS measurements were carried out in a buffer with a final NaCl concentration of 130 mM.

	K_d					
	Aspecific substrate			Specific substrate		
	IN·DNA	IN·DNA·p75	Fold increase in binding	IN·DNA	IN·DNA·p75	Fold increase in binding
	nM	*nM*		*nM*	*nM*	
HIV-1 IN	323 ± 12.5	50 ± 11	6.5	719 ± 52	31.6 ± 3.9	22.7
HIV-2 IN	370 ± 16.4	134 ± 9.7	2.8	730 ± 43	161 ± 12	4.5

protein of the transcription co-activator PC4 (25). LEDGF/p75 has also been shown to interact with components of the general transcription machinery and with the transcription activation domain of VP16. By coupling the promotion of DNA binding of the HIV-1 PIC to the transcriptional machinery, LEDGF/p75 may provide the missing link between integration and transcription.

In conclusion, we have shed light on the potential role of LEDGF/p75 during HIV replication. We provide clear evidence for a lentivirus-specific mechanism of tethering IN to DNA.

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REFERENCES

1. Coffin, J. M., Hughes, S. H., and Varmus, H. E. (1997) in *Retroviruses*, pp. 1–25, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Lewis, P. F., and Emerman, M. (1994) *J. Virol.* **68,** 510–516
3. Roe, T., Reynolds, T. C., Yu, G., and Brown, P. O. (1993) *EMBO J.* **12,**

2099–2108
4. Brown, P. O. (1997) *Curr. Top. Microbiol. Immunol.* **157,** 19–47
5. Craigie, R. (2001) *J. Biol. Chem.* **276,** 23213–23216
6. Asante-Appiah, E., and Skalka, A. M. (1997) *Antiviral Res.* **36,** 139–156
7. Asante-Appiah, E., and Skalka, A. M. (1999) *Adv. Virus Res.* **52,** 351–369
8. Engelman, A., Bushman, F. D., and Craigie, R. (1993) *EMBO J.* **12,** 3269–3275
9. Bushman, F. D., and Wang, B. (1994) *J. Virol.* **68,** 2215–2223
10. Engelman, A., and Craigie, R. (1992) *J. Virol.* **66,** 6361–6369
11. Jenkins, T. M., Engelman, A., Ghirlando, R., and Craigie, R. (1996) *J. Biol. Chem.* **271,** 7712–7718
12. Vercammen, J., Maertens, G., Gerard, M., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2002) *J. Biol. Chem.* **277,** 38045–38052
13. Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., De Clercq, E., and Debyser, Z. (2003) *J. Biol. Chem.* **278,** 372–381
14. Heuer, T. S., and Brown, P. O. (1998) *Biochemistry* **37,** 6667–6678
15. Cherepanov, P., Pluymers, W., Claeys, A., Proost, P., De Clercq, E., and Debyser, Z. (2000) *FASEB J.* **14,** 1389–1399
16. Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003) *J. Biol. Chem.* **278,** 33528–33539
17. Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M., Workman, J. L., and Crabtree, G. R. (1996) *EMBO J.* **15,** 5370–5382
18. Pruss, D., Reeves, R., Bushman, F. D., and Wolffe, A. P. (1994) *J. Biol. Chem.* **269,** 25031–25041
19. Muller, H. P., and Varmus, H. E. (1994) *EMBO J.* **13,** 4704–4714
20. Miller, M. D., and Bushman, F. D. (1995) *Curr. Biol.* **5,** 368–370
21. Kalpana, G. V., Marmon, S., Wang, W., Crabtree, G. R., and Goff, S. P. (1994) *Science* **266,** 2002–2006
22. Farnet, C. M., and Bushman, F. D. (1997) *Cell* **88,** 483–492
23. Violot, S., Hong, S. S., Rakotobe, D., Petit, C., Gay, B., Moreau, K., Billaud, G., Priet, S., Sire, J., Schwartz, O., Mouscadet, J. F., and Boulanger, P. (2003) *J. Virol.* **77,** 12507–12522
24. Lee, M. S., and Craigie, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1528–1533
25. Ge, H., Si, Y., and Roeder, R. G. (1998) *EMBO J.* **17,** 6723–6729
26. Ganapathy, V., Daniels, T., Casiano, C. A., Singh, D. P., Kimura, A., Chylack, L. T., Jr., Shinohara, T., Ohguro, N., Kikuchi, T., Sueno, T., Reddy, V. N., Yuge, K., and Fatma, N. (2003) *Autoimmun. Rev.* **2,** 290–297
27. Singh, D. P., Ohguro, N., Kikuchi, T., Sueno, T., Reddy, V. N., Yuge, K., Chylack, L. T., Jr., Shinohara, T., Fatma, N., and Kimura, A. (2000) *Biochem. Biophys. Res. Commun.* **267,** 373–381
28. Fatma, N., Singh, D. P., Shinohara, T., and Chylack, L. T., Jr. (2001) *J. Biol. Chem.* **276,** 48899–48907
29. Singh, D. P., Fatma, N., Kimura, A., Chylack, L. T., Jr., and Shinohara, T. (2001) *Biochem. Biophys. Res. Commun.* **283,** 943–955
30. Singh, D. P., Kimura, A., Chylack, L. T., Jr., Shinohara, T., Ohguro, N., Kikuchi, T., Sueno, T., Reddy, V. N., Yuge, K., and Fatma, N. (2000) *Gene* (*Amst.*) **242,** 265–273
31. Ge, H., Si, Y., and Wolffe, A. P. (1998) *Mol. Cell* **2,** 751–759
32. Lutzke, R. A., and Plasterk, R. H. (1998) *J. Virol.* **72,** 4841–4848
33. van den Ent, F. M., Vos, A., and Plasterk, R. H. (1999) *J. Virol.* **73,** 3176–3183
34. Vink, C., van der Linden, K. H., and Plasterk, R. H. (1994) *J. Virol.* **68,** 1468–1474
35. Chiu, R., and Grandgenett, D. P. (2003) *J. Virol.* **77,** 6482–6492
36. Wang, T., Piefer, A. J., and Jonsson, C. B. (2001) *J. Biol. Chem.* **276,** 14710–14717
37. Jonsson, C. B., Donzella, G. A., and Roth, M. J. (1993) *J. Biol. Chem.* **268,** 1462–1469
38. Villanueva, R. A., Jonsson, C. B., Jones, J., Georgiadis, M. M., and Roth, M. J. (2003) *Virology* **316,** 146–160
39. Van Craenenbroeck, E., and Engelborghs, Y. (1999) *Biochemistry* **38,** 5082–5088
40. Van Craenenbroeck, E., and Engelborghs, Y. (2000) *J. Mol. Recognit.* **13,** 93–100
41. Van Craenenbroeck, E., Vercammen, J., Matthys, G., Beirlant, J., Marot, C., Hoebeke, J., Strobbe, R., and Engelborghs, Y. (2001) *Biol. Chem.* **382,** 355–361
42. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 11.23–11.30, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
43. Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., and Poeschla, E. M. (2004) *J. Virol.* **78,** 9524–9537
44. Depienne, C., Roques, P., Creminon, C., Fritsch, L., Casseron, R., Dormont, D., Dargemont, C., and Benichou, S. (2000) *Exp. Cell Res.* **260,** 387–395
45. Depienne, C., Mousnier, A., Leh, H., Le Rouzic, E., Dormont, D., Benichou, S., and Dargemont, C. (2001) *J. Biol. Chem.* **276,** 18102–18107
46. Gallay, P., Hope, T., Chin, D., and Trono, D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9825–9830
47. Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., Sharova, N., Adzhubel, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stevenson, M. (1993) *Nature* **365,** 666–669
48. Gallay, P., Stitt, V., Mundy, C., Oettinger, M., and Trono, D. (1996) *J. Virol.* **70,** 1027–1032
49. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) *Cell* **101,** 173–185
50. Maertens, G., Cherepanov, P., Debyser, Z., Engelborghs, Y., and Engelman, A. (2004) *J. Biol. Chem.* **279,** 33421–33429
51. Devroe, E., Engelman, A., and Silver, P. A. (2003) *J. Cell Sci.* **116,** 4401–4408
52. Bushman, F. D. (2003) *Cell* **115,** 135–138
53. Wu, X., Li, Y., Crise, B., and Burgess, S. M. (2003) *Science* **300,** 1749–1751
54. Mitchell, R. S., Beitzel, B. F., Schroder, A. R., Shinn, P., Chen, H., Berry, C. C., Ecker, J. R., and Bushman, F. D. (2004) *PLoS Biol.* **2,** E234
55. Nishizawa, Y., Usukura, J., Singh, D. P., Chylack, L. T., Jr., and Shinohara, T. (2001) *Cell Tissue Res.* **305,** 107–114
56. Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R., and Bushman, F. (2002) *Cell* **110,** 521–529