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Biochemical and genetic analyses of integrase-interacting proteins lens epithelium-derived growth factor (LEDGF)/p75 and hepatoma-derived growth factor related protein 2 (HRP2) in preintegration complex function and HIV-1 replication

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Abstract

Human immunodeficiency virus type 1 (HIV-1) integrase (IN) functions in cells within the context of high molecular weight preintegration complexes (PICs). Lens epithelium-derived growth factor (LEDGF) transcriptional coactivator/p75 and hepatoma-derived growth factor related protein 2 (HRP2) tightly bind to HIV-1 IN and stimulate its integration activity in vitro. Here, we show that each recombinant host cell factor efficiently reconstitutes the in vitro activity of HIV-1 PICs disrupted for functional integration by pre-treatment with high concentrations of salt. Mutational analysis reveals that both the IN-binding and DNA-binding activities of LEDGF/p75 contribute to functional PIC reconstitution. We also investigate a role(s) for these proteins in HIV-1 infection by using short-interfering RNA. HIV-1 infection was essentially unaffected in HeLa-P4 cells depleted for LEDGF/p75, HRP2, or both proteins. We conclude that cells knocked-out for LEDGF/p75 and/or HRP2 will be useful genetic tools to address the roles of these host cell factors in HIV-1 replication.

Keywords: LEDGF/p75; HRP2; HIV-1; Integration; Integrase; PIC; RNAi

Introduction

The integration of reverse transcribed HIV-1 cDNA into a host cell chromosome is an essential step in the viral replication cycle (Englund et al., 1995; LaFemina et al., 1992; Sakai et al., 1993; Stevenson et al., 1990). Although purified HIV-1 integrase (IN) protein alone is sufficient to mediate simplified integration reactions in vitro (Bushman et al., 1990; Cherepanov et al., 1999; Sinha and Grandgenett, 2005; Sinha et al., 2002), integration in cells occurs within a large, highly ordered nucleoprotein complex termed the preintegration complex (PIC) that is derived from the viral core (Bowerman et al., 1989). The PIC not only provides an amenable environment for efficient catalysis of IN 3′ processing and DNA strand transfer activities, but is also expected to protect the cDNA from degradation and facilitate its transport into the nucleus. Recent evidence that HIV-1 preferentially integrates into actively transcribed genes (Mitchell et al., 2004; Schroder et al., 2002; Wu et al., 2003) has fuelled speculation that components of the PIC may also play a role in directing the virus to active transcription units during integration.

PICs isolated from acutely-infected cells can integrate their endogenous cDNA into an added target DNA in vitro (Brown et al., 1987; Farnet and Haseltine, 1990; Fujiwara and Mizuuchi, 1988; Lee and Coffin, 1991). The precise composition of HIV-1 PICs is largely unknown and is envisaged to change during maturation from reverse transcription within the cytoplasm to integration within the nucleus (Fassati and Goff,

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2001; Karageorgos et al., 1993). In addition to IN, the viral protein R (Vpr), matrix (MA), and reverse transcriptase (RT) proteins each associate with de novo-synthesized HIV-1 cDNA in cell extracts (Bukrinsky et al., 1993; Heinzinger et al., 1994; Karageorgos et al., 1993; Miller et al., 1997). A variety of cellular proteins, including the barrier-to-autointegration factor (BAF), high mobility group protein A1 (HMGA1), and lens epithelium-derived growth factor (LEDGF)/p75 also co-fractionate with HIV-1 PIC activity in vitro (Farnet and Bushman, 1997; Lin and Engelman, 2003; Llano et al., 2004b; Suzuki and Craigie, 2002). Integration activity can be disrupted by treating PICs with relatively high concentrations of salt, and BAF and HMGA1 were each purified through their ability to restore integration to salt-stripped retroviral PICs (Farnet and Bushman, 1997; Lee and Craigie, 1998). Neither BAF nor HMGA1 appears to directly bind to IN (Hindmarsh et al., 1999; Mansharamani et al., 2003). Instead, these proteins appear to gain access to PICs through their abilities to bind DNA, although for HIV-1, an interaction with MA is likely to contribute to BAF's recruitment (Mansharamani et al., 2003). See Engelman (2003) and Turlure et al. (2004) for reviews on the roles of host cell factors in retroviral DNA integration.

A separate category of HIV-1 integration co-factors, typified by LEDGF/p75, binds directly to IN (Cherepanov et al., 2003; Emiliani et al., 2005; Llano et al., 2004b; Turlure et al., 2004). This member of the hepatoma-derived growth factor (HDGF) family of proteins potently stimulated the activity of recombinant HIV-1 IN protein in in vitro integration assays (Cherepanov et al., 2003, 2004). When expressed on its own in the absence of other HIV-1 proteins, IN localizes to cell nuclei and LEDGF/p75 is a dominant cellular binding partner for HIV-1 IN under these conditions (Llano et al., 2004b; Maertens et al., 2003, 2004; Vanegas et al., 2005). RNA interference (RNAi)-mediated depletion of endogenous LEDGF/p75 redistributed IN from the nucleus to the cytoplasm (Llano et al., 2004b; Maertens et al., 2003). Likewise, expression of a nuclear localization-deficient mutant of LEDGF/p75 confined HIV-1 IN to the cell cytoplasm (Maertens et al., 2004). HIV-1 IN is a substrate for proteosomal degradation (Devroe et al., 2003; Mulder and Muesing, 2000) and binding to LEDGF/p75 protected IN from proteolysis in human cells (Llano et al., 2004a). In addition to HIV-1, LEDGF/p75 binds other lentiviral [HIV-2 (Busschots et al., 2005) and feline immunodeficiency virus (Llano et al., 2004b)] INs but fails to bind to either gammaretroviral Moloney murine leukemia virus (MoMLV) (Busschots et al., 2005; Llano et al., 2004b), alpharetroviral Rous sarcoma virus, or deltaretroviral human T-cell leukemia virus type 2 (Busschots et al., 2005) IN. Because lentiviruses target genes more often than do either gamma or alpharetroviruses, LEDGF/p75 is a PIC-associated factor that might play a particularly important role in helping HIV-1 to locate active transcription units during integration (see Engelman, 2005, for review).

The IN-binding domain (IBD) in LEDGF/p75, which resides within the C-terminal region of the protein (Cherepanov et al., 2004, 2005; Vanegas et al., 2005), is necessary although insufficient to stimulate HIV-1 IN activity in vitro (Cherepanov et al., 2004). A functional IBD also resides in a second HDGF family member, HDGF related protein 2 (HRP2) (Cherepanov et al., 2004; Vanegas et al., 2005). Akin to LEDGF/p75, purified HRP2 protein potently stimulated the in vitro integration activities of recombinant HIV-1 IN (Cherepanov et al., 2004). HRP2 predominantly localizes to the nucleus (Vanegas et al., 2005) and contains a PWWP (named for a signature amino acid sequence motif) domain that is present in many proteins that intimately associate with chromatin (Slater et al., 2003; Stec et al., 2000). However unlike LEDGF/p75, HRP2 does not appear to tether HIV-1 IN to chromatin when the viral protein is expressed from a synthetic gene (Vanegas et al., 2005).

To extend earlier biochemical studies on the roles of these proteins in integration, we assayed LEDGF/p75 and HRP2 for their abilities to reconstitute the integration activity of salt-stripped HIV-1 PICs. Each protein displayed robust activity in this assay. Furthermore, we show by mutational analysis that the ability of LEDGF/p75 to reconstitute activity to salt-stripped PICs relies on the presence of both DNA-binding and IN-binding motifs. To then assess a role for these proteins in HIV-1 infection using a genetic approach, we utilized short-interfering (si) RNAs to deplete either or both HDGF family member from cells that are susceptible to HIV-1. Of note, a previous RNAi-based study failed to reveal a role for LEDGF/p75 in HIV-1 replication (Llano et al., 2004b). Our results reveal normal infectivity levels in CD4 + HeLa cells reduced for LEDGF/p75, HRP2, or both proteins. Because our biochemical results further implicate a role(s) for these proteins in HIV-1 integration, we propose that cells ablated for LEDGF/p75 and/or HRP2 via genetic knockouts will be required to unequivocally address the roles of these IN-binding proteins in HIV-1 replication.

Results

Purified LEDGF/p75 and HRP2 efficiently restore integration activity to salt-stripped HIV-1 PICs

PICs isolated from HIV-1-infected cells can efficiently integrate their endogenous viral cDNA into an added target DNA in vitro (Chen et al., 1999; Farnet and Haseltine, 1990). Integration activity can be disrupted by incubation with relatively high concentrations of salt, and extracts of uninfected SupT1 T-cells reconstituted integration activity to salt-depleted HIV-1 PICs (Chen and Engelman, 1998; Farnet and Bushman, 1997). HMGA1 and BAF were each purified based on their ability to restore integration activity to salt-treated retroviral PICs (Farnet and Bushman, 1997; Lee and Craigie, 1998). In side-by-side tests, BAF displayed a greater specific activity than HMGA1 (Chen and Engelman, 1998; Suzuki and Craigie, 2002) while RNase A, utilized in some assays as an aspecific control, functioned similar to HMGA1 (Chen and Engelman, 1998).

Purified LEDGF/p75 and HRP2 proteins were previously shown to stimulate the in vitro integration activity of purified HIV-1 IN using recombinant DNA substrates that model the

ends of HIV-1 cDNA (Cherepanov et al., 2003, 2004), but neither of these proteins was previously tested for their potential to restore function to salt-depleted PICs. HIV-1 PICs isolated from acutely-infected cells were assayed for integration into ϕX174 target DNA as previously described (Fujiwara and Mizuuchi, 1988; Farnet and Haseltine, 1990; Chen et al., 1999). A portion of the preparation was treated with 1.2 M KCl on ice to strip potential co-factors essentially as previously described (Chen and Engelman, 1998); as some PIC loss was encountered during subsequent spin column chromatography and ultrafiltration steps, approximately 6-fold more material was analyzed by agarose gel electrophoresis and Southern blotting after salt-stripping (Fig. 1, compare lanes 7 and 8 to lanes 5 and 6). The initial PIC preparation before salt treatment converted about 29% of its 9.7 kb HIV-1 cDNA substrate into the 15.1 integration product (Fig. 1, compare lanes 1 and 2) and, as previously established (Chen and Engelman, 1998; Farnet and Bushman, 1997), salt-stripping effectively ablated in vitro PIC function (Fig. 1, lane 4). Incubating salt-stripped PICs with purified LEDGF/p75 protein prior to addition of ϕX174 target DNA restored starting levels of HIV-1 integration activity (Fig. 1, lane 3).

We next tested other purified proteins (HRP2, BAF, and RNase A) as well as specific LEDGF/p75 mutant proteins (see below) alongside wild-type LEDGF/p75 to assess their activities in the PIC reconstitution assay. To facilitate this analysis, we employed a real-time quantitative (RQ)-PCR assay that was recently developed to assess HIV-1 PIC activity in vitro (Lu et al., 2005b). This assay was particularly useful for assessing multiple protein samples, as reconstitution activity was reproducibly detected using just 7% of the level of salt-stripped material required for detection by Southern blotting (200 μl versus 3 ml). Due to the noted loss of material incurred during salt-stripping and subsequent purification steps, integration activity was normalized to the total levels

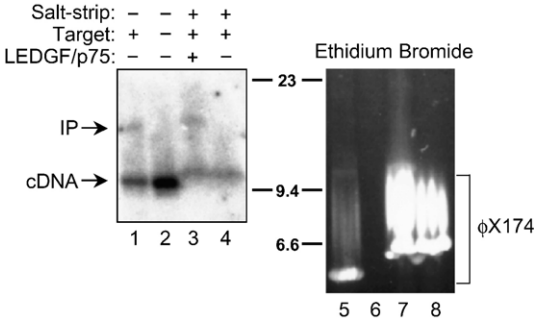

Fig. 1. Reconstitution of salt-stripped HIV-1 PIC activity with LEDGF/p75. Lane 1, PIC integration activity prior to salt-stripping; lane 2, level of initial HIV-1 cDNA substrate in 0.5 ml of crude cytoplasmic extract; lane 3, reconstitution of PIC activity following addition of LEDGF/p75; lane 4, salt-stripped PICs incubated with target DNA in the absence of LEDGF/p75. The migration positions of unreacted substrate DNA and the covalent product of HIV-1 integration are marked cDNA and IP, respectively. The positions of molecular mass standards in kb are indicated to the right of the blot. An ethidium stain of the agarose gel used for Southern blotting is shown in lanes 5–8. The 9 μg of ϕX174 target DNA utilized in salt-stripped PIC integration reactions resulted in somewhat retarded DNA mobilities in lanes 3, 4, 7, and 8 relative to lanes 1, 2, 5, and 6, respectively.

of viral cDNA present in the different samples. Integration activity after reconstitution was expressed as the percentage of PIC function prior to salt depletion. LEDGF/p75 restored about 43% of the level of starting PIC activity in this experiment (Fig. 2A); replicate measurements (n = 7) revealed LEDGF/p75 displayed between 40% and 100% activity under these assay conditions (data not shown). HRP2 restored about 39% of starting integration activity under conditions where BAF and RNase A restored approximately 15% and 2.4% of PIC function, respectively (Fig. 2A).

LEDGF/p75 IN-binding and DNA-binding activities contribute to functional PIC reconstitution

We next investigated LEDGF/p75 functions that might contribute to PIC reconstitution activity. The protein contains two evolutionarily-conserved subdomains, an N-terminal PWWP domain (residues 1–93) and the IBD (residues 347–429) (Cherepanov et al., 2004) (Fig. 2B). The IBD is necessary but not sufficient to stimulate purified HIV-1 IN activity in vitro (Cherepanov et al., 2004). LEDGF/p75 residue Asp-366 is essential for the interaction with IN, and full-length LEDGF/p75 containing the D366N amino acid substitution displayed approximately 8% of wild-type LEDGF/p75 activity in the IN stimulation assay (Cherepanov et al., 2005; data not shown). Purified LEDGF/p75 binds to DNA in vitro (Singh et al., 2001; Busschots et al., 2005) and we recently determined that the nuclear localization signal (NLS, residues 146–156; Fig. 2B) and a dual copy of the AT-hook DNA-binding motif that spans residues 178–197 mediates the binding of LEDGF/p75 to DNA (F. Turlure, G. Maertens, P. Cherepanov, A. Engelman, submitted for publication). Based on these observations, the following purified LEDGF/p75 mutant proteins were tested: 347–471, which contains the IBD, binds IN, but does not appreciably stimulate its activity (Cherepanov et al., 2004); D366N, which fails to bind IN (Cherepanov et al., 2005); 1–266, which lacks the IBD but retains DNA-binding activity (submitted for publication); mutL1, which contains 12 amino acid substitutions in the NLS and AT-hook motifs and displays roughly 3% residual DNA-binding activity (submitted for publication); and mutL1/D366N, which lacks both IN and DNA-binding functions (Fig. 2B).

The 347–471 protein restored about 5.5% of starting PIC function indicating that, akin to the result utilizing mini-HIV substrate DNA (Cherepanov et al., 2004), IN-binding on its own is insufficient to reconstitute significant salt-stripped PIC activity (Fig. 2A). In contrast, the D366N missense mutant displayed near-wild type activity in repeat experiments (32% relative integration activity in Fig. 2A). Based on this, we hypothesized that LEDGF/p75 DNA-binding activity was likely to contribute to its functional PIC reconstitution activity. As 1–266 restored only about 8% of starting PIC function (Fig. 2A), DNA-binding in the absence of IN-binding could only marginally restore PIC function. Full-length mutL1 retained about 40% of wild-type LEDGF/p75 function, indicating that DNA-binding played a modest role in LEDGF/p75 function under conditions where the wild-type affinity for IN is

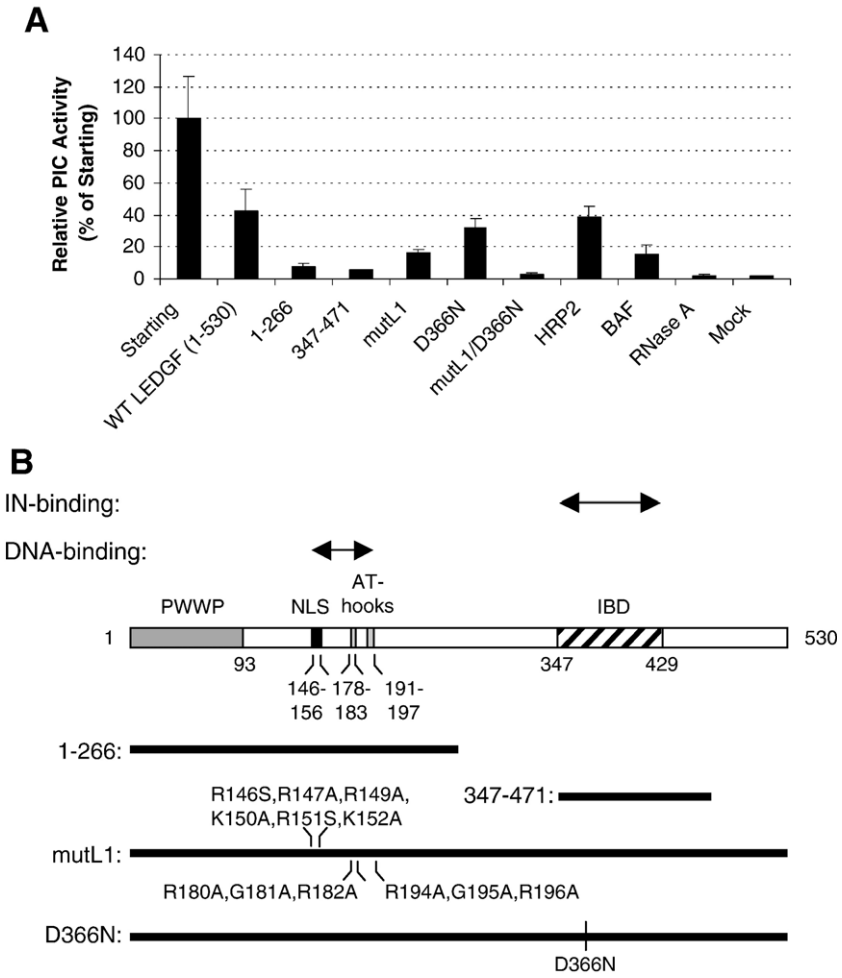

Fig. 2. Reconstitution of salt-stripped PIC activity using LEDGF/p75, HRP2, BAF, RNase A, and LEDGF/p75 mutant proteins. (A) Starting, level of PIC activity prior to salt-stripping, normalized to level of HIV-1 cDNA substrate. Mock, level of integration activity following incubation with BGF buffer alone. LEDGF/p75 was observed to reconstitute 2- to 3-fold more activity than BAF following three independent sets of HIV-1 infection, PIC isolation, and salt-stripping experiments. Error bars indicate variation obtained following duplicate RQ-PCR assays. Similar results were obtained following independent sets of salt-stripping and reconstitution assays. (B) Schematic diagram of LEDGF/p75, highlighting functional domains. The DNA-binding mutant protein mutL1 carries 6 amino acid substitutions in the NLS as well as the substitutions of Ala–Ala–Ala for the invariant Arg–Gly–Arg sequences that comprise the heart of the AT-hook DNA-binding motif (Reeves, 2001).

maintained (Fig. 2A). As the mutL1/D366N combination mutant failed to appreciably reconstitute PIC function (about 3% relative integration activity), we conclude that DNA-binding and IN-binding play synergistic roles in LEDGF/p75 PIC reconstitution activity (Fig. 2A).

Reducing intracellular levels of LEDGF/p75 does not appreciably affect HIV-1 infectivity

To address the roles of LEDGF/p75 and HRP2 in HIV-1 replication, protein levels in HeLa-P4 cells (Charneau et al., 1992) were reduced using transient delivery of siRNA prior to infection with replication-competent HIV-1_{IIIB}. This CD4-positive indicator line was chosen because the cells (i) were readily transfectable with siRNA and (ii) contain an integrated β-galactosidase reporter gene under the control of the Tat-responsive viral U3 promoter. This latter trait afforded a convenient and quantitative screen for HIV-1 infectivity, which was assessed following in situ staining with 5-bromo-4-chloro-3-indolyl-b-D-galactoside (Kimpton and Emerman, 1992).

Eight siRNAs that targeted different regions of LEDGF/p75 were screened for their potential to suppress mRNA. Cells transfected on 2 successive days were lysed 72 h after the first transfection, and LEDGF/p75 levels were measured by quantitative (q) RT-PCR. Two previously-described siRNAs, L3 (Maertens et al., 2003) and 21 [based on a shRNA expression vector (Devroe and Silver, 2002)] (Fig. 3A), reduced LEDGF/p75 mRNA approximately 27- and 14-fold, respectively (Fig. 3B). Co-transfection of 21 and L3 siRNAs did not increase the knock-down beyond that observed with L3 siRNA alone (Fig. 3B, 21/L3).

Llano et al. (2004b) previously-described 293T and Jurkat cell-based lines that stably express short-hairpin (sh) RNA and potently down-regulate LEDGF/p75 expression. Normalized levels of total input RNA was analyzed in side-by-side qRT-PCR assays to compare the extent of LEDGF/p75 down-regulation in these cells versus that achieved with the transient

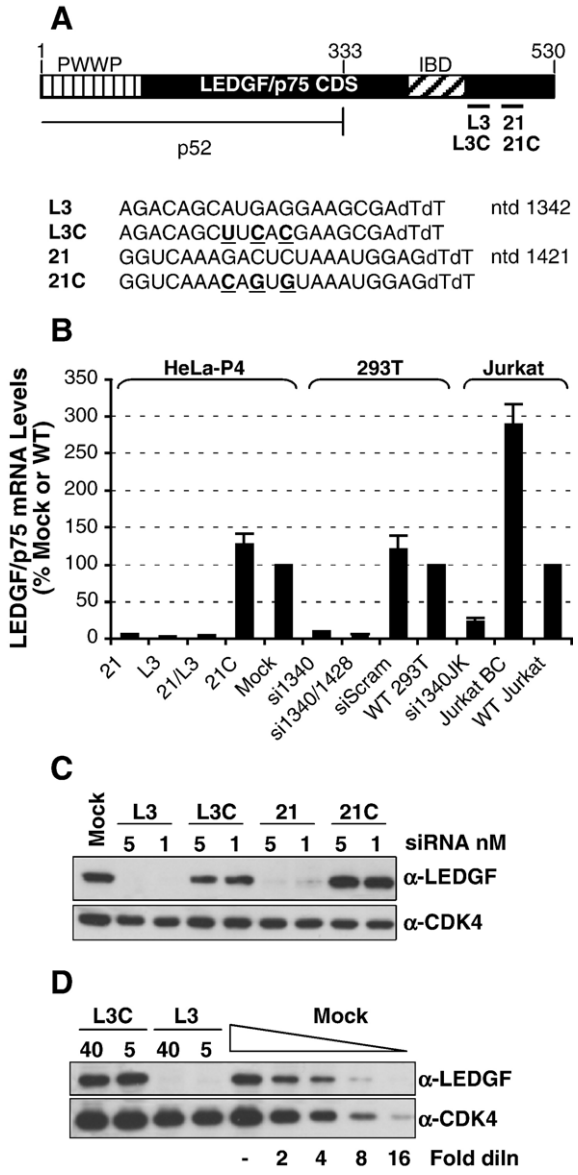

Fig. 3. siRNA design and knock-down of LEDGF/p75. (A) Sense-strand sequences, starting nucleotide (ntd), and relative positions of L3 and 21 siRNAs and matched controls (L3C and 21C) along the LEDGF/p75 coding sequence (CDS). Nucleotides in the matched controls that differed from L3 and 21 are in bold and underlined. Relative positions of the alternative splice variant LEDGF/p52, the conserved PWWP domain, and the IBD are shown; numbers atop the CDS indicate amino acid position. (B) Relative LEDGF/p75 mRNA levels following transfection of HeLa-P4 cells with the indicated siRNAs at 5 nM. The 21/L3 sample contained 10 nM total siRNA. Previously-described si1340, si1340/1428, siScram (derived from 293T cells), and si1340JK Jurkat cells (Llano et al., 2004b) were included for comparison. The JurkatBC cell line expressed shRNA-resistant LEDGF/p75 in addition to si1340 shRNA (Llano et al., 2004b). Error bars indicate variance obtained in duplicate qRT-PCR assays. Similar results were obtained following an independent set of RNA isolation and qRT-PCR assays. (C) LEDGF/p75 protein levels at 72 h post-transfection with the indicated concentrations of siRNA. Blotting for CDK4 confirmed that similar levels of total cell protein (10 μg) were analyzed across samples. (D) Semi-quantitative western analysis of the L3 siRNA-specific knock-down. Numbers above lanes 1–4 are siRNA concentrations in nM.

siRNA delivery system developed here. Whereas the level of mRNA down-regulation in 293T-based si1340 cells was approximately 12-fold, si1340/1428 cells, which expressed two different targeting shRNAs, harbored about 17-fold less LEDGF/p75 message than parental 293T cells (Fig. 3B). Control siScram cells, which expressed a non-targeting shRNA, contained a normal level of LEDGF/p75 mRNA (Fig. 3B). Whereas the level of LEDGF/p75 message in si1340JK cells was reduced about 4.4-fold, a derivative of this cell line engineered to express a shRNA-resistant form of LEDGF/p75 contained about 3-fold more message than parental Jurkat cells (Fig. 3B). We note that si1340 shRNA was based on L3 siRNA and si1428 shRNA overlapped with the 21 siRNA used here (identical stretch of 16 nucleotides). Given the caveats that siRNA was used in HeLa-P4 cells and shRNA was expressed in 293T and Jurkat cells, we concluded that on average, siRNA was approximately 1.6-fold more effective than shRNA at reducing LEDGF/p75 mRNA levels (Fig. 3B).

Reducing siRNA concentration from 5 nM to 1 nM revealed undetectable and barely detectable levels of LEDGF/p75 protein in cells transfected with L3 and 21 siRNAs, respectively (Fig. 3C). As observed at the mRNA level, control siRNAs yielded negligible effects on LEDGF/p75 protein levels (Figs. 3B–D). Semi-quantitative western blotting revealed that L3 siRNA mediated at least an 8-fold reduction in the steady-state level of LEDGF/p75 protein (Fig. 3D).

Mock- and siRNA-transfected cells cultured with HIV-1_{IIIB} for 36 h were stained for β-galactosidase expression, and the resulting blue cells were counted by light microscopy. Cells transfected with either 21 or 21C siRNA supported levels of HIV-1 infectivity that were nearly-identical to mock-treated cells (Fig. 4A). Likewise, cells treated with 1 nM or 5 nM of L3 siRNA did not reveal any appreciable affect on the level of HIV-1 infection. In contrast, cells transfected with 40 nM L3 siRNA reproducibly supported 2- to 3-fold less infection than control cells (Fig. 4A and data not shown). Similar results were obtained when single-round HIV-1_{NL4-3} was used in place of replication-competent HIV-1_{IIIB} (data not shown), indicating that results were independent of the viral strain or source of virus (transiently-transfected 293T cell supernatant in the case of single round HIV-1_{NL4-3} versus supernatant of chronically-infected MOLTIIIB T-cells).

Because (i) cells transfected with 21 siRNA supported near-wild type levels of infection (Fig. 4A) and (ii) the level of LEDGF/p75 knock-down appeared similar in cells transfected with L3 siRNA at 40 nM or 5 nM (Fig. 3D), we were concerned that the infectivity defect in cells transfected with 40 nM L3 siRNA could be caused by affects unrelated to the reduction in LEDGF/p75 protein. To assess the fitness of cells under different conditions of siRNA treatment, parallel cultures of cells utilized in Fig. 4A were analyzed for their proliferative capacity using a commercially-available kit. Cells treated with L3 siRNA at 40 nM were approximately 2.4-fold reduced in their capacity to proliferate as compared to mock-transfected cells (Fig. 4B). Similar results were observed using either trypan blue staining or a second commercially-available kit that measured cell viability (data not shown). We noted that the proliferative status of cell cultures closely mirrored the level at which they supported HIV-1 infection (compare Fig. 4B to A). Together with the observations that 40 nM and 5 nM L3 siRNA

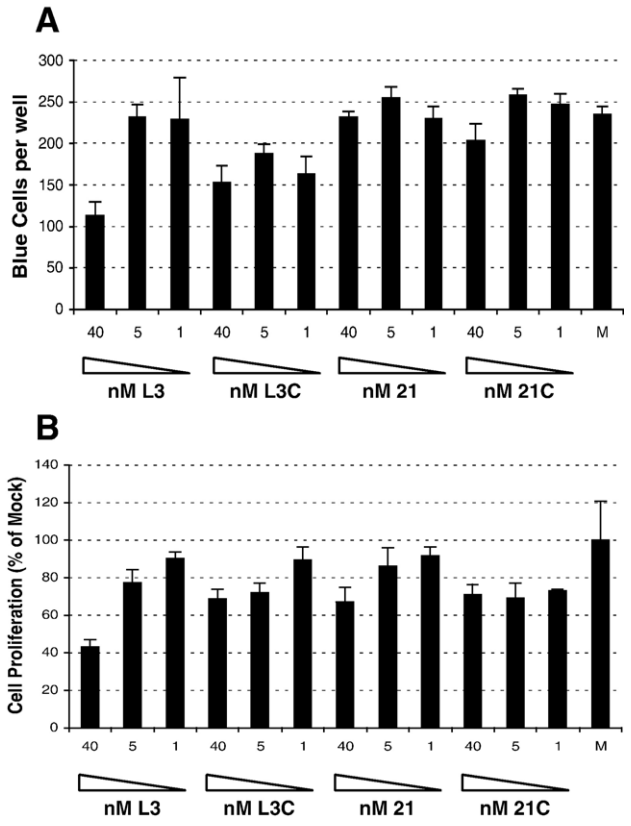

Fig. 4. HIV-1 infectivity and proliferative status of siRNA-treated cells. (A) Infectivity was quantified by averaging the total number of blue cells in quadruplicate cultures 36 h post-infection (p.i.) with HIV-1_{IIIB}. (B) Cell proliferation was assessed at 24 h p.i. M, mock.

appeared to reduce LEDGF/p75 to similar levels (Fig. 3D) and that an infectivity defect was not seen in cells treated with 21 siRNA despite effective knock-down of LEDGF/p75 protein (Figs. 3C, 4A, and data not shown), the proliferation data indicated that reductions in infectivity were more likely due to decreased cell fitness rather than to the specific depletion of LEDGF/p75 from cells.

Wild type levels of HIV-1 infection in cells reduced for both LEDGF/p75 and HRP2

We recently determined that HRP2 was a second member of the HDGF family of proteins that contained an IBD and potently stimulated the in vitro integration activity of recombinant HIV-1 IN (Cherepanov et al., 2004). Because cells depleted for LEDGF/p75 using shRNA vectors (Llano et al., 2004b) or chemically-synthesized siRNA (Figs. 3B–D and 4A) supported near-wild type levels of HIV-1 infection, one hypothesis was that HRP2 effectively played the role of LEDGF/p75 in LEDGF/p75-depleted cells (Cherepanov et al., 2004). To experimentally address this hypothesis, siRNAs were screened for their ability to reduce intracellular HRP2 levels. Of thirteen siRNAs tested by qRT-PCR, only two significantly reduced HRP2. Of these, Dh4 siRNA (Fig. 5A) was the least cytotoxic (data not shown) and therefore was used in subsequent experiments. HRP2 mRNA was reduced approximately 12-fold in HeLa-P4 cells transfected with Dh4 siRNA at three different concentrations although notably, control Dh4C3 siRNA at 40 nM also moderately reduced the message (Fig. 5B). Consistent with this observation, Dh4 and Dh4C3 siRNAs negatively-impacted cell viability and proliferation when either was used at 40 nM (data not shown). Subsequent experiments therefore utilized Dh4 siRNA at 5 nM.

Because an anti-HRP2 antibody was not available, the potential for Dh4 siRNA to knock-down HRP2 protein levels was assessed by co-transfecting siRNAs with a haemagglutinin (HA)-tagged HRP2 expression vector (Cherepanov et al., 2004). Previous analyses of HA-HRP2 expression in human 293T cells revealed that it migrated significantly more slowly in sodium dodecyl sulfate (SDS)-polyacrylamide gels than recombinant HRP2 protein (Cherepanov et al., 2004), indicating that HRP2 might undergo post-translational modification in animal cells. Analysis of the HRP2 protein sequence using the NetPhos 2.0 Server (Blom et al., 1999) revealed numerous potential sites of Ser, Tyr, and Thr phosphorylation and because of this, the migration pattern of HA-HRP2 was investigated following treatment with calf intestinal phosphatase (CIP). Because the electrophoretic mobility of HA-HRP2 shifted after CIP treatment, we concluded that the protein was indeed phosphorylated in human cells (Fig. 5C). HA-HRP2-containing lysates were subsequently treated with CIP prior to electrophoresis and Western blotting.

Co-transfecting Dh4 siRNA with the HA-HRP2 expression vector ablated HRP2 detection (Fig. 5D, lane 1), indicating that Dh4 siRNA likely reduced intracellular levels of endogenous HRP2 protein. Of note, HA-HRP2 was efficiently expressed following co-transfection with each of the Dh4-matched control siRNAs (Fig. 5D, lanes 2–4).

HIV-1_{IIIB} infectivity was assessed in HeLa-P4 cells transfected with Dh4 siRNA or L3 siRNA alone, in combination, or with matched control siRNAs. Neither Dh4 siRNA nor L3 siRNA negatively impacted cell proliferation (Fig. 6A) or viability (not shown) when used separately at 5 nM or in combination (10 nM total siRNA). Following co-transfection with Dh4 and L3 siRNAs, HRP2 and LEDGF/p75 mRNAs were reduced to levels similar to those observed following treatment with individual siRNAs (Figs. 3B, 5B, and data not shown). HIV-1 infectivity was not significantly altered in HeLa-P4 cells transfected with Dh4 siRNA or co-transfected with Dh4 and L3 siRNAs versus cells transfected with matched control siRNAs (Fig. 6B). Of note, HRP2 and LEDGF/p75 mRNA levels remained suppressed during the course of HIV-1 infection (data not shown).

Discussion

Efficient reconstitution of HIV-1 PIC activity with purified LEDGF/p75 or HRP2 protein

Previous experiments established that LEDGF/p75 was a dominant cellular binding partner of HIV-1 IN when the virus protein was expressed on its own in human cells (Cherepanov et al., 2003; Llano et al., 2004a, 2004b; Maertens et al., 2003,

2004; Turlure et al., 2004). Consistent with a potential role for the IN-LEDGF/p75 interaction in HIV-1 biology, LEDGF/p75 was found to associate with HIV-1 PICs (Llano et al., 2004b). Treating HIV-1 PICs with relatively high concentrations of salt was previously shown to ablate their in vitro integration activity, and extracts of uninfected T-cells restored function to salt-depleted PICs (Chen and Engelman, 1998; Farnet and Bushman, 1997). These observations were some of the first to indicate that host cell proteins likely played important roles in HIV-1 integration (reviewed in Engelman, 2003; Turlure et al., 2004). LEDGF/p75 and HRP2 potently stimulated the in vitro integration activity of recombinant HIV-1 IN protein (Cherepanov et al., 2003; 2004), but neither protein was previously tested for its ability to restore function to salt-depleted HIV-1 PICs. Here, we show that purified LEDGF/p75 protein functions quite efficiently in this assay. LEDGF/p75 could restore integration activity to a level similar to that present prior to salt-stripping (Fig. 1 and data not shown). Similar analyses using HMGA1 (Farnet and Bushman, 1997) and BAF (Chen and Engelman, 1998) reported reconstitution of approximately 50% of initial PIC function by each of these factors, indicating that LEDGF/p75 is particularly active under these assay conditions. Mutational analyses revealed that both the IN-

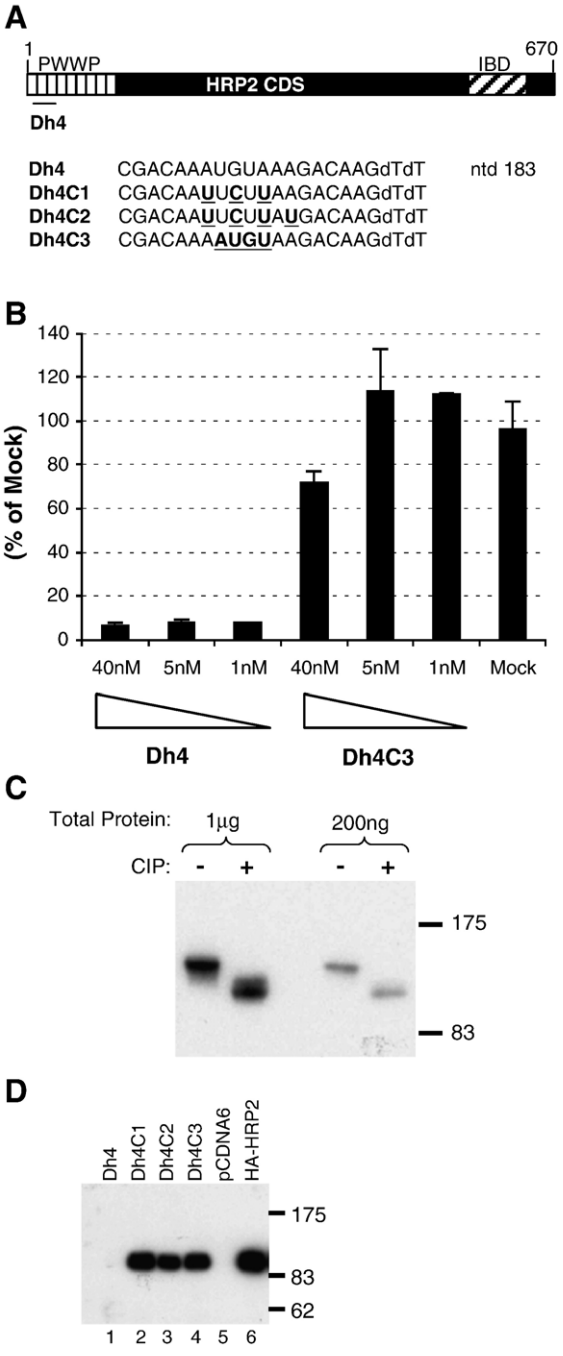

Fig. 5. HRP2 knock-down via siRNA. (A) Sense-strand sequence, starting nucleotide (ntd), and relative position of Dh4 siRNA and matched controls (Dh4C1, Dh4C2, and Dh4C3) along the HRP2 coding sequence (CDS). Nucleotides in control sequences that differed from Dh4 siRNA are bold and underlined. The PWWP domain and IBD are indicated. (B) Relative HRP2 mRNA levels following transfection of the indicated siRNAs at 40 nM, 5 nM, or 1 nM. Duplicate RNA samples harvested at 48 h post-transfection were analyzed by qRT-PCR. (C) HA-HRP2 is phosphorylated in vivo. Lysates were either treated (+) with CIP or left untreated (−) prior to electrophoresis of 1 µg or 200 ng of total cell protein, as indicated. The migration positions of molecular mass standards in kDa are indicated to the right of the gel. (D) HA-HRP2 protein levels following co-transfection with siRNA. Total cell protein recovered 36 h after co-transfecting cells with the pCPHA-HRP2 expression vector and either Dh4 siRNA (lane 1) or indicated matched control siRNAs (lanes 2–4). Control transfections included pCDNA6 (the plasmid from which pCPHA-HRP2 was derived; lane 5) and pCPHA-HRP2 without added siRNA (lane 6). Migration positions of molecular mass standards in kDa are indicated to the right.

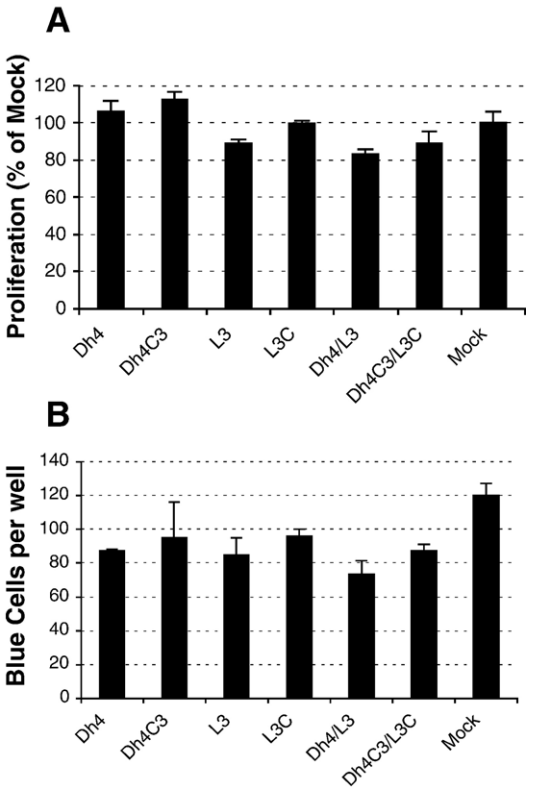

Fig. 6. Proliferative and infectivity statuses of cells following treatment with different siRNAs. (A) Cell proliferation was quantified 24 h p.i. with the indicated siRNAs. (B) Average numbers of blue cell counts were determined in quadruplicate cultures 36 h p.i. with HIV-1_{IIIB}.

binding and DNA-binding functions of LEDGF/p75 contributed to its activity in the PIC reconstitution assay (Fig. 2).

Normal levels of HIV-1 infection in cells depleted for LEDGF/p75 and/or HRP2

Despite the mass of biochemical data implicating a role for LEDGF/p75 in HIV-1 replication, previous attempts to reduce the intracellular level of LEDGF/p75 protein using shRNA vectors failed to ascribe a genetic link between endogenous levels of LEDGF/p75 protein and HIV-1 infection (Llano et al., 2004b). The IBD in LEDGF/p75 was subsequently determined to reside in another protein, HRP2 (Cherepanov et al., 2004). We therefore envisioned two mutually non-exclusive reasons for why HIV-1 replicated in cells knocked-down for LEDGF/p75 expression: (i) residual levels of endogenous LEDGF/p75 protein within these cells were sufficient to support wild type levels of integration and/or (ii) replacement of LEDGF/p75 function by the IBD-containing HRP2.

To address these hypotheses, HeLa-P4 cells were subjected to transient delivery of siRNA. Cells containing approximate 27-fold and 10-fold reductions in LEDGF/p75 mRNA and protein, respectively (Figs. 3B, C, and D), supported normal levels of HIV-1 infection (Fig. 4A). When L3 siRNA was used at 40 nM, however, we routinely observed 2- to 3-fold reductions in HIV-1 infectivity (Fig. 4A). This effect was not recapitulated using L3 siRNA at 5 nM or 21 siRNA at 5 nM or 40 nM, even though these treatments likewise elicited potent reductions in LEDGF/p75 message and protein (Figs. 3B–D and data not shown). Side-by-side infectivity and cell proliferation measurements indicated that the infection defect observed at 40 nM L3 siRNA was likely due to an impaired ability for the transfected cells to grow (Fig. 4). These observations highlight the need to confirm biological phenotypes with multiple siRNAs, as previously recommended (Anonymous, 2003; Huppi et al., 2005; Jackson et al., 2003; Jackson and Linsley, 2004). Our findings agree with those of Llano et al. (2004b) who reported that 293T and Jurkat cells harboring reduced levels of LEDGF/p75 protein supported normal levels of HIV-1 infection.

In terms of whether residual levels might suffice to support wild type levels of HIV-1 integration, we note that HeLa-P4 cells harbor a relative abundance of LEDGF/p75 protein. Using serial dilutions of purified LEDGF/p75 protein and semi-quantitative western blotting, we estimated approximately 4×10^5 molecules of LEDGF/p75 protein per cell (Fig. 7). Based on this, we conclude that approximately 25,000 copies of LEDGF/p75 are likely to persist under our most effective knock-down conditions (Fig. 3D). Given the relatively high affinity of IN for LEDGF/p75, which appears to be on the order of 10 nM (P. Cherepanov and A. Engelman, unpublished observations), we speculate that relatively high residual levels of LEDGF/p75 might function to support normal levels of HIV-1 integration. Given the limitations of RNAi technologies to erase residual levels of target proteins from cells, the relatively high copy number of LEDGF/p75 protein in

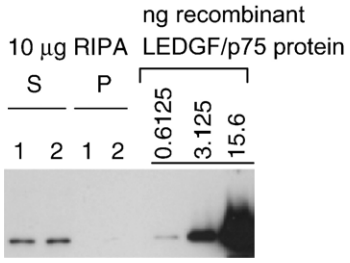

Fig. 7. Estimation of the LEDGF/p75 copy number in HeLa-P4 cells. Duplicate supernatant (S) and pellet (P) fractions from 3×10^4 cells were analyzed by Western blotting alongside a 5-fold dilution series of recombinant LEDGF/p75 protein. Densitometric scanning revealed approximately 1.2 ng of LEDGF/p75 in each of the first two lanes, which, based on a molecular mass of 60,103 Da (Cherepanov et al., 2005), equated to approximately 400,645 protein molecules per cell.

susceptible target cells, and the relatively strong interaction between LEDGF/p75 and HIV-1 IN, we propose that genetic knock-out cell lines will be required to unequivocally address the role of LEDGF/p75 in HIV-1 replication.

To investigate the hypothesis that HRP2 might provide an important function for HIV-1 in cells harboring reduced levels of LEDGF/p75, HRP2 was targeted by siRNA (Fig. 5A). Cells containing approximately 12-fold less HRP2 mRNA than control cells (Fig. 5B) supported near wild type levels of HIV-1 infectivity (Fig. 6B). Normal levels of HIV-1 infection were also observed in cells reduced for both HRP2 and LEDGF/p75 (Fig. 6B). Because an antibody against HRP2 was unavailable, our findings must be interpreted with the limitation that we were unable to measure any potential reduction in endogenous HRP2 protein upon siRNA treatment. We did however demonstrate significant reductions in ectopically-expressed HA-HRP2 upon cotransfection with Dh4 siRNA (Fig. 5D).

Our results with HRP2 are perhaps not all that surprising in light of recent findings that ectopically-expressed HRP2 yielded a pattern of intra-nuclear localization that differed from LEDGF/p75 (Vanegas et al., 2005). Whereas LEDGF/p75 tightly associated with chromatin (Cherepanov et al., 2003; Emiliani et al., 2005; Maertens et al., 2003; Nishizawa et al., 2001; Vanegas et al., 2005), HRP2 appeared diffuse throughout the nucleus and did not strongly associate with DNA. Furthermore, the relative affinity of the HRP2-IN interaction in human cells appeared to be much lower than the LEDGF/p75-IN interaction (Cherepanov et al., 2004; Vanegas et al., 2005). Although these findings do not discount a role for HRP2 in the early events of HIV-1 replication, the close association of LEDGF/p75 with chromatin and the relative strength of the IN-LEDGF/p75 interaction suggest that LEDGF/p75 is more likely than HRP2 to play a role in HIV-1 integration/replication.

Because of the inability of RNAi technology to completely erase targeted gene products from cells, we have initiated projects to genetically ablate LEDGF/p75 and HRP2 from cells. Until these reagents become available, it may prove difficult to ascertain by genetic means whether the plethora of encouraging biochemical data for these host factors translates to clear biological roles in HIV-1 replication.

Materials and methods

Expression plasmids and recombinant proteins

Vectors for expressing HA-tagged and untagged HRP2 in animal and bacterial cells, respectively, were previously described (Cherepanov et al., 2004). Recombinant LEDGF/p75 protein with an N-terminal His_6-tag was expressed in *Escherichia coli* from pFT-1-LEDGF, which was derived from pRSETB (Invitrogen Corp.) by amplifying LEDGF/p75 sequences from pCP-Nat75 (Maertens et al., 2003) with *Bgl*II-tagged 5′-GCGCAGATCT**CTGGAAGTTCTGTTCCAGGGGCCC**GGATCCATCACTCGCGATTTCAAAC-CTGGAGACC and *Eco*RI-tagged 5′-CGCGAATTCCTAGTT-ATCTAGTGTAGAATCC (restriction enzyme sites underlined) using *Pfu*Ultra DNA polymerase (Stratagene) under conditions recommended by the manufacturer. The bold bases in the *Bgl*II-tagged primer encode for LEVLFQGP, which is the recognition site for PreScission Protease (Amersham Biosciences Corp.). Amplified DNA digested with *Bgl*II and *Eco*RI was ligated with *Bam*HI/*Eco*RI-digested pRSETB.

Cells harboring pFT-1-LEDGF were grown at 32 °C to an optical density of 0.8–0.9 at 600 nm, at which time isopropyl-thio-β-D-galactopyranoside was added to the final concentration of 0.5 mM. Cells grown for an additional 4 h at 28 °C were harvested, frozen at −80 °C, and thawed cells were lysed in buffer A [1 M NaCl, 25 mM Tris–HCl pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM imidazole, EDTA-free Complete protease inhibitor (Roche Diagnostics)]. Sonicated lysates clarified by centrifugation were incubated with Ni-NTA agarose beads (QIAGEN) for 1 h at 4 °C, and bound proteins were eluted in buffer A containing 200 mM imidazole. LEDGF/p75-containing fractions were pooled and the His-tag was removed by incubating overnight at 4 °C with 4 U of PreScission Protease (Amersham Biosciences Corp.) per mg of protein. Cleaved LEDGF/p75 diluted 5-fold with 50 mM NaH_2PO_4, pH 7.2 (to reduce the NaCl concentration to 200 mM) was loaded onto a HiTrap SP Sepharose column (Amersham Biosciences Corp.) and was eluted with a linear gradient of NaCl from 0.2 to 1 M in 50 mM NaH_2PO_4 pH 7.2. Pooled LEDGF/p75-containing fractions were further purified by gel filtration using a Superdex 200 column (Amersham Biosciences Corp.) in 120 mM NaCl, 25 mM Tris–HCl pH 7.4. LEDGF/p75-containing fractions were pooled, concentrated using a Centricon-10 concentrator (Millipore Corp.), supplemented with glycerol to 10% (wt/vol), flash frozen in liquid N_2, and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

HRP2, the LEDGF/p75 IBD (residues 347–471), and LEDGF/D366N were purified as previously described (Cherepanov et al., 2004, 2005). The 1–266 deletion mutant expressed as a fusion to glutathione *S*-transferase from pGEX-6P3 (Amersham Biosciences Corp.) was purified essentially as described for previous LEDGF/p75 deletion mutant proteins (Cherepanov et al., 2004). The GST tag was removed by cleavage with PreScission Protease as outlined above. The mutL1 protein, expressed from a pFT-1-LEDGF-based vector, was purified as described above for wild-type LEDGF/p75.

Human BAF was purified following its expression in bacteria essentially as previously described (Harris and Engelman, 2000) with the addition that re-folded BAF dimers were purified by gel filtration on a Superdex 200 column operated in BGF buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 50 mM β-mercaptoethanol, 10% [wt/vol] glycerol). RNase A was from QIAGEN.

PIC isolation, salt-stripping, and integration assays

PICs isolated at 7 h p.i. from cytoplasmic extracts of MOLTIIIB and SupT1 cell co-cultures as described (Chen et al., 1999) were adjusted to contain 7% sucrose (wt/vol), snap-frozen in liquid N_2, and stored at −80 °C. Salt-stripping was performed essentially as previously described (Brooun et al., 2001; Chen and Engelman, 1998). Briefly, samples (700 μl) in isotonic buffer K (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 1 mM dithiothreitol [DTT], 20 μg/ml aprotinin, 0.025% [wt/vol] digitonin) were thawed on ice and incubated with an equal volume of no-salt buffer K (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT) for 30 min on ice before centrifuging at 8000 × *g* for 20 min at 4 °C. Pelleted material resuspended in 700 μl of high-salt buffer K (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1.2 M KCl, 1 mM DTT) was incubated on ice for 30 min. High salt-treated PICs were partially purified by Sepharose CL-4B (Amersham Biosciences Corp.) spin column chromatography (Chen et al., 1999). Eluate concentrated to approximately 87.5 μl by centrifugation in a Microcon-100 ultrafiltration unit (Millipore Corp.) at 14,000 × *g* was readjusted to isotonic salt concentration by adding 612.5 μl of no-salt buffer K. For reconstitution, purified proteins (800 nM final) diluted into 20 μl of BGF buffer were incubated with 180 μl salt-stripped PICs in the presence of 0.1 mg/ml bovine serum albumin for 1 h on ice. Reconstitution assays were scaled-up for Southern blotting to a final volume of 3 ml. Integration into ϕX174 target DNA (3 μg/ml) proceeded for 45 min at 37 °C, and integration activity was quantified by PhosphorImager using ImageQuant version 1.2 as the percent of total HIV-1 cDNA converted to product.

For RQ-PCR-based PIC assays, plasmid pTZ18U/PL (3 μg/ml) was utilized instead of ϕX174 as an integration target. Primers and cycling parameters were the same as in Lu et al. (2005b) with the exception that 23 cycles were utilized in first round reactions. Percent functional reconstitution was defined as the level of integration activity as compared to starting PICs (180 μl + 20 μl BGF buffer). Activities of reconstituted versus starting samples were normalized to the total levels of HIV-1 cDNA in the different samples, as described (Lu et al., 2005b).

Cell lines

HeLa-P4 cells (Charneau et al., 1992) and 293T cells were cultured in Dulbecco's modified Eagle Medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml

streptomycin (DMEM). SupT1 T-cells (Smith et al., 1984), Jurkat T-cells (Weiss et al., 1984), and chronically-infected MOLTIIIB T-cells (Farnet and Haseltine, 1990) were cultured in RPMI 1640 media containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (RPMI).

A number of cell lines expressing shRNA were a generous gift from Dr. Eric Poeschla (Mayo Clinic, Rochester, Minnesota). si1340 and siScram cells, derived from 293T cells, were grown in DMEM containing hygromycin B (200 μg/ml); the media for 293T-based siL1340/1428 cells additionally contained puromycin at 3 μg/ml. JurkatBC and si1340JK cells, derived from Jurkat T-cells, were grown in RPMI containing 800 μg/ml hygromycin B.

Viruses and infections

Replication-competent HIV-1_{IIIB} collected from high-density cultures of MOLTIIIB cells was passed through a 0.45-μm filter and stored at −80 °C. Single-round viruses carrying HIV-1_{NL4-3} envelope glycoproteins were derived by co-transfecting 293T cells with pNLX.Luc(R-) and pNLXE7 at the ratio of 30:1 using calcium-phosphate as previously described (Lu et al., 2005a; Sambrook et al., 1989). Culture supernatants collected 48 h post-transfection were filtered prior to use. All viruses were titered on HeLa-P4 cells essentially as previously described (Engelman et al., 1995; Kimpton and Emerman, 1992). Cells (6.7×10^3/well) plated in 96 well plates 24 h before infection were stained for β-galactosidase activity using standard protocols (Kimpton and Emerman, 1992) between 36 h and 48 h p.i. Cell viability was assessed either by trypan blue exclusion or the CellTiter-Blue Cell Viability Assay (Promega Corp.). Cell proliferation was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp.).

Design, synthesis, and transfection of siRNAs

Double-stranded siRNAs were purchased from Dharmacon Research. L3 (Maertens et al., 2003) and 21 [based on a shRNA expression vector (Devroe and Silver, 2002)] were previously-described LEDGF/p75-specific siRNAs. Whereas the sense-strand sequence of L3 siRNA was 5′-AGACAGCAUGAGGAAGCGAdTdT, the 21 sequence was 5′-GGUCAAAGACUCUAAAUGGAGdTdT. The following controls that differed from antagonistic siRNAs by three internal base changes were also utilized: L3C siRNA (5′-AGACAGCUUCACGAAGCGAdTdT; mutated bases underlined) and 21C (5′-GGUCAAACAGUGUAAAUGGAGdTdT). The sense-strand sequence of Dh4 siRNA targeting HRP2 was 5′-CGACAAAUGUAAAGACAAGdTdT. In this case three different control siRNAs, containing three or four base changes, were utilized: Dh4C1 (5′-CGACAAUUCUUAAGACAAGdTdT), Dh4C2 (5′-CGACAAUUCUUAUGACAAGdTdT), and Dh4C3 (5′-CGACAAAAUGUAAGACAAGdTdT).

HeLa-P4 cells (2×10^5) plated in 6-well trays the day before transfection were transfected twice on successive days with indicated concentrations of siRNA using oligofectamine (Invitrogen) essentially as described by the manufacturer (see http://www.invitrogen.com/content/sfs/protocols/sirna_oftsf_proc.pdf). Cells were co-transfected just once with siRNA and plasmid DNA using $CaPO_4$.

Protein extraction and western blotting

Proteins were extracted on ice from HeLa-P4 cells for 15 min using RIPA buffer (20 mM HEPES pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2 mM EDTA, complete protease inhibitor [Roche Diagnostics]), and protein concentration was quantified using the D_c Protein Assay (Bio-Rad Laboratories). LEDGF/p75 and cyclin-dependent kinase 4 (CDK4, which served as a loading control) were detected by western blotting as described (Devroe and Silver, 2002).

To determine the approximate LEDGF/p75 copy number in HeLa-P4 cells, duplicate samples (2×10^6 cells) following extraction with RIPA buffer were centrifuged, and 10 μg of total protein in supernatant fractions (equating to 3×10^4 cells) were compared by Western blotting alongside a matched volume of pelleted fractions resuspended in protein sample buffer and a 5-fold dilution series of recombinant LEDGF/p75 protein. Quantitation was performed using Quantify One version 4.1.1 software (Bio-Rad Laboratories).

Lysates containing HA-HRP2 were treated with 5 U of CIP (New England Biolabs Inc.) for 15 min at 30 °C before loading onto 6% Tris-glycine gels (Invitrogen). Proteins transferred to polyvinylidene difluoride membrane using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad Laboratories) were detected using anti-HA.11 clone 16B12 antibody (Covance Research Products, Inc.) and the ECL Plus Western Blotting kit (Amersham Biosciences Corp.).

RNA extraction and qRT-PCR

RNA extracted from cells using the RNeasy Mini Kit (QIAGEN) was quantified by spectrophotometry. Primers for qRT-PCR were designed to span intron/exon boundaries. Primers for amplifying cyclophilin A (CypA) were: 5′-TTCATCTGCACTGCCAAGAC and 5′-TGGTCTTGCCATTCCTGGAC. LEDGF/p75-specific primers were 5′-AAGCTAGAGAAGGAACAAAC and 5′-TTGCTGTCTTCATTGCTCTC and HRP2 primers were 5′-AAGTTTGCCCTAAAGGTCGAC and 5′-GCTTTGTAACGGCGAATCT.

Semi-quantitative standard curves were constructed by analyzing 5-fold dilutions of RNA (from 25 ng to 0.04 ng) extracted from mock-transfected cells or parental cell lines. PCR was performed with the QuantiTect SYBR Green RT-PCR kit (QIAGEN) using either 5 ng (for CypA) or 25 ng (LEDGF/p75 and HRP2) of template RNA in a final volume of 30 μl under conditions recommended by the manufacturer. Reactions conducted in a DNA Engine Opticon thermal cycler (MJ Research Inc.) were: 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Melting curve analyses (from 55 °C to 90 °C, read every 1 °C) were conducted to verify that each primer pair

predominantly amplified a single DNA species. Values of unknown samples were normalized to the CypA content of RNA extracts using the OpticonMONITOR Analysis Software version 2.01 supplied by the manufacturer.

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References

Anonymous, 2003. Whither RNAi? Nat. Cell Biol. 5, 489–490.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294, 1351–1362.

Bowerman, B., Brown, P.O., Bishop, J.M., Varmus, H.E., 1989. A nucleoprotein complex mediates the integration of retroviral DNA. Genes Dev. 3, 469–478.

Brooun, A., Richman, D.D., Kornbluth, R.S., 2001. HIV-1 preintegration complexes preferentially integrate into longer target DNA molecules in solution as detected by a sensitive, polymerase chain reaction-based integration assay. J. Biol. Chem. 276, 46946–46952.

Brown, P.O., Bowerman, B., Varmus, H.E., Bishop, J.M., 1987. Correct integration of retroviral DNA in vitro. Cell 49, 347–356.

Bukrinsky, M.I., Sharova, N., McDonald, T.L., Pushkarskaya, T., Tarpley, W.G., Stevenson, M., 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. U.S.A. 90, 6125–6129.

Bushman, F.D., Fujiwara, T., Craigie, R., 1990. Retroviral DNA integration directed by HIV integration protein in vitro. Science 249, 1555–1558.

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.

Charneau, P., Alizon, M., Clavel, F., 1992. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. 66, 2814–2820.

Chen, H., Engelman, A., 1998. The barrier-to-autointegration protein is a host factor for HIV type 1 integration. Proc. Natl. Acad. Sci. U.S.A. 95, 15270–15274.

Chen, H., Wei, S.Q., Engelman, A., 1999. Multiple integrase functions are required to form the native structure of the human immunodeficiency virus type I intasome. J. Biol. Chem. 274, 17358–17364.

Cherepanov, P., Surratt, D., Toelen, J., Pluymers, W., Griffith, J., De Clercq, E., Debyser, Z., 1999. Activity of recombinant HIV-1 integrase on mini-HIV DNA. Nucleic Acids Res. 27, 2202–2210.

Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., De Clercq, E., Debyser, Z., 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J. Biol. Chem. 278, 372–381.

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.

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.

Devroe, E., Silver, P.A., 2002. Retrovirus-delivered siRNA. BMC Biotechnol. 2, 15.

Devroe, E., Engelman, A., Silver, P.A., 2003. Intracellular transport of human immunodeficiency virus type 1 integrase. J. Cell Sci. 116, 4401–4408.

Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D., Vandekerckhove, L., Moisant, F., Ben-Slama, L., Witvrouw, M., Christ, F., Rain, J.C., Dargemont, C., Debyser, Z., Benarous, R., 2005. Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. J. Biol. Chem. 280, 25517–25523.

Engelman, A., 2003. The roles of cellular factors in retroviral integration. Curr. Top. Microbiol. Immunol. 281, 209–238.

Engelman, A., 2005. The ups and downs of gene expression and retroviral DNA integration. Proc. Natl. Acad. Sci. U.S.A. 102, 1275–1276.

Engelman, A., Englund, G., Orenstein, J.M., Martin, M.A., Craigie, R., 1995. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J. Virol. 69, 2729–2736.

Englund, G., Theodore, T.S., Freed, E.O., Engleman, A., Martin, M.A., 1995. Integration is required for productive infection of monocyte-derived macrophages by human immunodeficiency virus type 1. J. Virol. 69, 3216–3219.

Farnet, C.M., Bushman, F.D., 1997. HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro. Cell 88, 483–492.

Farnet, C.M., Haseltine, W.A., 1990. Integration of human immunodeficiency virus type 1 DNA in vitro. Proc. Natl. Acad. Sci. U.S.A. 87, 4164–4168.

Fassati, A., Goff, S.P., 2001. Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. J. Virol. 75, 3626–3635.

Fujiwara, T., Mizuuchi, K., 1988. Retroviral DNA integration: structure of an integration intermediate. Cell 54, 497–504.

Harris, D., Engelman, A., 2000. Both the structure and DNA binding function of the barrier-to-autointegration factor contribute to reconstitution of HIV type 1 integration in vitro. J. Biol. Chem. 275, 39671–39677.

Heinzinger, N.K., Bukrinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.A., Gendelman, H.E., Ratner, L., Stevenson, M., Emerman, M., 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. U.S.A. 91, 7311–7315.

Hindmarsh, P., Ridky, T., Reeves, R., Andrake, M., Skalka, A.M., Leis, J., 1999. HMG protein family members stimulate human immunodeficiency virus type 1 and avian sarcoma virus concerted DNA integration in vitro. J. Virol. 73, 2994–3003.

Huppi, K., Martin, S.E., Caplen, N.J., 2005. Defining and assaying RNAi in mammalian cells. Mol. Cell 17, 1–10.

Jackson, A.L., Linsley, P.S., 2004. Noise amidst the silence: off-target effects of siRNAs? Trends Genet. 20, 521–524.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21, 635–637.

Karageorgos, L., Li, P., Burrell, C., 1993. Characterization of HIV replication complexes early after cell-to-cell infection. AIDS Res. Hum. Retrovir. 9, 817–823.

Kimpton, J., Emerman, M., 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. 66, 2232–2239.

LaFemina, R.L., Schneider, C.L., Robbins, H.L., Callahan, P.L., LeGrow, K., Roth, E., Schleif, W.A., Emini, E.A., 1992. Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. J. Virol. 66, 7414–7419.

Lee, Y.M., Coffin, J.M., 1991. Relationship of avian retrovirus DNA synthesis to integration in vitro. Mol. Cell. Biol. 11, 1419–1430.

Lee, M.S., Craigie, R., 1998. A previously unidentified host protein protects retroviral DNA from autointegration. Proc. Natl. Acad. Sci. U.S.A. 95, 1528–1533.

Lin, C.W., Engelman, A., 2003. The barrier-to-autointegration factor is a

component of functional human immunodeficiency virus type 1 preintegration complexes. J. Virol. 77, 5030–5036.

Llano, M., Delgado, S., Vanegas, M., Poeschla, E.M., 2004a. Lens epithelium-derived growth factor/p75 prevents proteasomal degradation of HIV-1 integrase. J. Biol. Chem. 279, 55570–55577.

Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., Poeschla, E.M., 2004b. 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.

Lu, R., Limon, A., Ghory, H.Z., Engelman, A., 2005a. Genetic analyses of DNA-binding mutants in the catalytic core domain of human immunodeficiency virus type 1 integrase. J. Virol. 79, 2493–2505.

Lu, R., Vandegraaff, N., Cherepanov, P., Engelman, A., 2005b. Lys-34, Dispensable for integrase catalysis, is required for preintegration complex function and human immunodeficiency virus type 1 replication. J. Virol. 79, 12584–12591.

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.

Maertens, G., Cherepanov, P., Debyser, Z., Engelborghs, Y., Engelman, A., 2004. Identification and characterization of a functional nuclear localization signal in the HIV-1 integrase interactor LEDGF/p75. J. Biol. Chem. 279, 33421–33429.

Mansharamani, M., Graham, D.R., Monie, D., Lee, K.K., Hildreth, J.E., Siliciano, R.F., Wilson, K.L., 2003. Barrier-to-autointegration factor BAF binds p55 Gag and matrix and is a host component of human immunodeficiency virus type 1 virions. J. Virol. 77, 13084–13092.

Miller, M.D., Farnet, C.M., Bushman, F.D., 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J. Virol. 71, 5382–5390.

Mitchell, R.S., Beitzel, B.F., Schroder, A.R., Shinn, P., Chen, H., Berry, C.C., Ecker, J.R., Bushman, F.D., 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol. 2, E234.

Mulder, L.C., Muesing, M.A., 2000. Degradation of HIV-1 integrase by the N-end rule pathway. J. Biol. Chem. 275, 29749–29753.

Nishizawa, Y., Usukura, J., Singh, D.P., Chylack Jr., L.T., Shinohara, T., 2001. Spatial and temporal dynamics of two alternatively spliced regulatory factors, lens epithelium-derived growth factor (ledgf/p75) and p52, in the nucleus. Cell Tissue Res. 305, 107–114.

Reeves, R., 2001. Molecular biology of HMGA proteins: hubs of nuclear function. Gene 277, 63–81.

Sakai, H., Kawamura, M., Sakuragi, J., Sakuragi, S., Shibata, R., Ishimoto, A., Ono, N., Ueda, S., Adachi, A., 1993. Integration is essential for efficient gene expression of human immunodeficiency virus type 1. J. Virol. 67, 1169–1174.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainville, NY.

Schroder, A.R., Shinn, P., Chen, H., Berry, C., Ecker, J.R., Bushman, F., 2002. HIV-1 integration in the human genome favors active genes and local hotspots. Cell 110, 521–529.

Singh, D.P., Fatma, N., Kimura, A., Chylack Jr., L.T., 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.

Sinha, S., Grandgenett, D.P., 2005. Recombinant human immunodeficiency virus type 1 integrase exhibits a capacity for full-site integration in vitro that is comparable to that of purified preintegration complexes from virus-infected cells. J. Virol. 79, 8208–8216.

Sinha, S., Pursley, M.H., Grandgenett, D.P., 2002. Efficient concerted integration by recombinant human immunodeficiency virus type 1 integrase without cellular or viral cofactors. J. Virol. 76, 3105–3113.

Slater, L.M., Allen, M.D., Bycroft, M., 2003. Structural variation in PWWP domains. J. Mol. Biol. 330, 571–576.

Smith, S.D., Shatsky, M., Cohen, P.S., Warnke, R., Link, M.P., Glader, B.E., 1984. Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Res. 44, 5657–5660.

Stec, I., Nagl, S.B., van Ommen, G.J., den Dunnen, J.T., 2000. The PWWP domain: a potential protein–protein interaction domain in nuclear proteins influencing differentiation? FEBS Lett. 473, 1–5.

Stevenson, M., Stanwick, T.L., Dempsey, M.P., Lamonica, C.A., 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. 9, 1551–1560.

Suzuki, Y., Craigie, R., 2002. Regulatory mechanisms by which barrier-to-autointegration factor blocks autointegration and stimulates intermolecular integration of Moloney murine leukemia virus preintegration complexes. J. Virol. 76, 12376–12380.

Turlure, F., Devroe, E., Silver, P.A., Engelman, A., 2004. Human cell proteins and human immunodeficiency virus DNA integration. Front. Biosci. 9, 3187–3208.

Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M., Poeschla, E., 2005. Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering. J. Cell Sci. 118, 1733–1743.

Weiss, A., Wiskocil, R.L., Stobo, J.D., 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. J. Immunol. 133, 123–128.

Wu, X., Li, Y., Crise, B., Burgess, S.M., 2003. Transcription start regions in the human genome are favored targets for MLV integration. Science 300, 1749–1751.