

Lens Epithelium-derived Growth Factor/p75 Prevents Proteasomal Degradation of HIV-1 Integrase*

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Manuel Llano[‡], Sharon Delgado[‡], Maria Vanegas^{‡§}, and Eric M. Poeschla^{‡¶}

From the [‡]Molecular Medicine Program and [§]Department of Immunology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

The transcriptional coactivator lens epithelium-derived growth factor (LEDGF)/p75 acts as a chromatin tethering factor for human immunodeficiency virus type 1 (HIV-1) integrase protein, determining its nuclear localization and its tight association with nuclear DNA. Here we identify a second function for the LEDGF/p75-integrase interaction. We observed that stable introduction of HIV-1 integrase (IN) transcription units into cells made stringently LEDGF/p75-deficient by RNAi resulted in much lower steady state levels of IN protein than introduction into LEDGF/p75 wild type cells. The same LEDGF/p75-dependent disparity was observed for feline immunodeficiency virus IN. However, IN mRNA levels were equivalent in the presence and absence of LEDGF/p75. A post-translational mechanism was confirmed when the half-life of HIV-1 IN protein was found to be much shorter in LEDGF/p75-deficient cells. Proteasome inhibition fully countered this extreme instability, increasing IN protein levels to those seen in LEDGF/p75 wild type cells and implicating proteasomal destruction as the main cause of IN instability. Consistent with these data, increased ubiquitinated HIV-1 IN was found in the LEDGF/p75 knock-down cells. Moreover, restoration of LEDGF/p75 to knocked down clones rescued HIV-1 IN stability. Subcellular fractionation showed that HIV-1 IN is exclusively cytoplasmic in LEDGF/p75-deficient cells, but mainly nuclear in LEDGF/p75 wild type cells, and that cytoplasmic HIV-1 IN has a shorter half-life than nuclear HIV-1 IN. However, using LEDGF proteins defective for nuclear localization and IN interaction, we further determined that protection of HIV-1 IN from the proteasome requires neither chromatin tethering nor nuclear residence. Protection requires only interaction with LEDGF/p75, and it is independent of the subcellular localization of the IN-LEDGF complex.

Lens epithelium-derived growth factor (LEDGF/p75)¹ is a transcriptional coactivator that interacts with the general transcription machinery (1, 2). Roles as a regulator of cellular stress responses and as an autoantigen have been proposed

(3–5). LEDGF/p75, which is 530 amino acids in length, and LEDGF/p52, a 333-amino acid splice variant, have the same N-terminal 325 amino acids. The two LEDGF proteins have wholly different C termini (8 amino acids in the case of p52 versus 205 amino acids for p75 (1, 6)). Although LEDGF/p75 is less active as a coactivator than LEDGF/p52, it is much more abundant in cells (7, 8).

IN proteins of human immunodeficiency virus type 1 (HIV-1) and other lentiviruses localize to nuclei when expressed in the absence of other viral proteins (7, 9–13). Recently, LEDGF/p75 has been shown to interact with lentiviral IN proteins directly, to be required for their nuclear targeting, and to tether them to nuclear chromatin (7, 14, 15). HIV-1 and feline immunodeficiency virus (FIV) IN proteins precisely colocalize with LEDGF/p75 in cells. RNAi against LEDGF/p75 produces a strikingly complete and stable shift of both lentiviral integrase proteins from the nucleus to the cytoplasm (7). In addition, LEDGF/p75 was also shown to be a component of lentiviral preintegration complexes (7). The failure of LEDGF/p52 to interact with IN (7, 15) implicates the 205-amino acid LEDGF/p75 C terminus in this interaction. The interaction is specific to lentiretroviruses (7) suggesting the possibility of a role in their distinctive biological features. Association between LEDGF/p75 and HIV-1 integrase has been confirmed to be direct in experiments with recombinant proteins (15).

26 S proteasomes are multicatalytic, multisubunit proteases that comprise a major protein-degrading apparatus active in both the cytoplasm (16) and the nucleus (17–22) of eukaryotic cells. This selective system is known to be essential for degrading misfolded proteins. However, its scope extends to fast turnover of normal but short-lived key regulatory proteins, *e.g.* transcriptional regulators (Jun, Fos, p53, I κ B-NF κ B, MyoD, c-Myc), cell cycle proteins (cyclins, p21^{Cip1}), and rate-limiting metabolic pathway enzymes (ornithine decarboxylase) (23, 24). In most cases, proteasome substrates are modified by covalent attachment of ubiquitin (Ub), a 76-amino acid polypeptide. Ub is bound through an isopeptide link established between its C terminus and the ϵ -NH₂ groups of lysine residues present either on the target protein or on a previously attached Ub molecule. Proteins with oligomers of four or more Ub molecules are targeted for degradation into short peptides by the 20 S catalytic subunit of the proteasome (24). However, ubiquitin-independent proteasome degradation also occurs. Ornithine transcarbamylase, which is the most rapidly turned over mammalian enzyme, and p21^{Cip1} are among the ubiquitin-independent exceptions (25–29).

HIV-1, simian immunodeficiency virus, and Moloney murine leukemia virus Gag have been shown to be monoubiquitinated in the virion (30). Inhibition of the proteasome interferes with HIV-1 Gag processing and HIV-1 and Rous sarcoma virus virion release and maturation (31). Inhibition of the proteasome in target cells also enhances HIV-1 infectivity, suggesting

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[¶] To whom correspondence should be addressed: Guggenheim 18, Mayo Clinic College of Medicine, 200 First St. S.W., Rochester, MN 55905. Tel.: 507-284-3178; Fax: 507-266-2122; E-mail: emp@mayo.edu.

¹ The abbreviations used are: LEDGF, lens epithelium-derived growth factor; IN, integrase; HIV-1, human immunodeficiency virus type 1; FIV, feline immunodeficiency virus; WT, wild type; Ub, ubiquitin; shRNA, short hairpin RNA; RNAi, RNA interference; RT, reverse transcriptase; mAb, monoclonal antibody; NLS, nuclear localization signal; HA, hemagglutinin.

that the proteasome may target incoming virions or host proteins involved in the early steps of the infection (32). In this regard, HIV-1 IN has been shown to undergo proteasome-mediated degradation (33). A ubiquitin-HIV-1 IN fusion protein was also demonstrated to be unstable in transiently transfected cells, and stability was enhanced by proteasome inhibition (34). However, HIV-1 IN can be expressed in cells at high levels provided Rev dependence is taken into account (7).

HIV-1 encoded proteins are known to manipulate the proteasome system to bring about specific degradation of producer cell host proteins that inhibit production of infectious virus. Vpu triggers proteasome degradation of the viral receptor CD4 (35). Vif links the proteasome to the cellular factor APOBEC3G, facilitating its degradation. In the absence of Vif, APOBEC3G incorporates into the virion and catalyzes lethal point mutations in the viral genome during reverse transcription (36–39). The present work identifies a third example of manipulation of the proteasome system by an HIV-1 protein. In cell lines stringently knocked down for LEDGF/p75 by RNAi, we observed markedly reduced steady state levels of HIV-1 and FIV IN expression despite equivalent mRNA levels. Further study showed that LEDGF/p75 acts to protect lentiviral INs from proteasomal degradation, a process that requires interaction of IN with LEDGF/p75 but not nuclear residence of the complex. IN interaction with LEDGF/p75 provides an example of an HIV-1 protein that manipulates proteasome degradation in an inhibitory rather than a stimulatory fashion.

EXPERIMENTAL PROCEDURES

LEDGF/p75 Plasmids—pLEDGF/p75siMut expresses wild type LEDGF/p75. The cDNA contains seven synonymous mutations in the short hairpin RNA (shRNA) target site to permit expression in cells knocked down for the endogenous protein (7). This plasmid was then used to generate mutants lacking the nuclear localization signal (pLEDGF/p75siMut.NLS[−]) or amino acids 340–417 (pLEDGF/p75siMut.Δ340–417) by overlap extension PCR.² pUbiquitin-HA (41) was a gift from D. Bohmann (University of Rochester Medical Center). Wild type LEDGF/p75 and LEDGF/p52 expression plasmids (15) were kindly provided by Z. Debyser (Rega Institute, Leuven).

Cell Culture, Transfections, Generation, and Drug Treatment of LH, SH, LF, and SF Cell Lines and Clones—si1340 cells (abbreviated as “L” cells) stably express a highly effective anti-LEDGF/p75 shRNA (7). siScram (abbreviated as “S” cells) cells stably express a control shRNA (the Ambion “scramble” small interfering RNA); each was derived from 293T cells for use in previous subcellular localization studies (7). L cells and S cells were further stably transfected with HIV-1 IN expression plasmids, generating the LH and SH cell lines, respectively. Analogous FIV IN-expressing lines, LF and SF, were also derived from L cells and S cells, respectively. For this stable lentiviral HIV-1 and FIV IN expression, plasmids with monocistronic transcription units were used, each containing an IN open reading frame with an internal ribosome entry site-linked selectable marker gene (puromycin resistance, *pac*) immediately downstream. The promoter is the human cytomegalovirus immediate early gene promoter, located upstream of the IN cDNA. In addition, the HIV-1 and FIV IN cDNAs are C-terminally Myc epitope-tagged, which has previously been shown to have no effect on either the subcellular localization or the interaction with LEDGF/p75 (7). Prior to transfection, these IN-internal ribosome entry site-*pac* plasmids were linearized at a restriction site in the prokaryotic backbone. Selection and maintenance was in puromycin 3 μ g/ml; lines were derived from at least 1000 separate stable colonies. From these four polyclonal founder lines (LH, SH, LF, SF), single-cell clones were then established by limiting dilution, picked randomly, and assigned sequential numbers (e.g. LH1, LH2, LH3); clones from 96-well plates having fewer than 15 positive wells were chosen to assure clonality. Transfections of derived cell lines were performed by the calcium phosphate coprecipitation method with a total of 2 μ g of DNA per well of a six-well plate or 1 μ g of DNA per chamber in a two-chamber LabTek II glass chamber slide (Nalge Nunc, Naperville, IL). Briefly, cells were transfected 24 h after being plated in 2 ml of medium at 0.45×10^6 cells/well or 1 ml of medium at 0.8×10^6 cells/chamber. After 14–16 h, the transfection mix

was replaced with fresh culture medium. Cells were harvested or used for indirect immunofluorescence or Western blotting 40–48 h after the transfection mix was added. Stable cell lines were incubated for 18 h with MG132 (50 μ M). For transiently transfected cells the drug was also added for 18 h, starting 24 h after transfection. Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin, and streptomycin; 200 μ g/ml hygromycin (L and S cells) and combined hygromycin and 3 μ g/ml puromycin (LH, SH, LF, SF cells) were added as indicated.

Immunoblotting—Cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, and 150 mM Tris-HCl, pH 8.0) plus protease inhibitors (Complete-mini, Roche Applied Science). Proteins (30 μ g/lane) were resolved in SDS-10% polyacrylamide gels and transferred to Immobilon P membranes (Millipore). Blocked membranes were incubated overnight at 4° C with anti-Myc epitope monoclonal antibody (mAb, clone 9E10, Covance), anti- α -tubulin mAb (clone B-5-1-2, Sigma), anti-LEDGF/p75 mAb (BD Biosciences), or a rabbit antibody to HIV-1 IN in Tris-buffered saline, 5% nonfat milk plus 0.05% Tween 20. After washing, membranes were incubated with the appropriate horseradish peroxidase-tagged secondary antibody. Bound antibodies were detected by ECL (Amersham Biosciences).

Immunoprecipitation—Cells cotransfected with pUbiquitin-HA were lysed in radioimmune precipitation assay buffer supplemented with 50 μ M MG132, 10 mM *N*-ethylmaleimide (Sigma) and protease inhibitors. 200 μ l of cell lysate were incubated with 30 μ l of agarose beads coupled to a rat anti-HA mAb (Roche Applied Science) or with a rabbit anti-HIV IN antibody plus 30 μ l of magnetic beads coupled to protein A (DynaL Biotech) for 1 h at 4° C. The beads were washed three times with lysis buffer containing MG132 and *N*-ethylmaleimide followed by boiling for 7 min in Laemmli buffer containing β -mercaptoethanol. The immunoprecipitated proteins were then evaluated by immunoblotting using a rabbit anti-HIV-1 IN antibody or an anti-HA rat monoclonal antibody (clone 3F10, Roche Applied Science).

Confocal Immunofluorescence Microscopy—Indirect immunofluorescence detection of Myc-tagged proteins was performed by confocal fluorescence microscopy with anti-Myc epitope mAb (clone 9E10) or a polyclonal anti-Myc antibody (Santa Cruz Biotechnology) when costained. Endogenous LEDGF/p75 was detected using anti-LEDGF/p75 mAb. Cells grown in LabTek II chamber slides (Nalge Nunc Naperville, IL) were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min at 37° C, washed with phosphate-buffered saline, and then permeabilized with ice-cold methanol for 2 min at room temperature. Fixed cells were blocked in phosphate-buffered saline with 10% fetal calf serum and 20 mM ammonium chloride for 30 min at room temperature and then incubated with the appropriate antibodies followed by Alexa-488- or Texas Red-conjugated goat anti-mouse antibody or goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (Molecular Probes).

Protein Half-life Determination—LH and LF clones were treated with 5 μ g/ml cycloheximide for 30 min at 37° C and transferred to ice until analysis. Cells were lysed in radioimmune precipitation assay buffer (total fraction), or cytoplasmic and nuclear cell fractions were isolated as described previously (42). IN was detected in total or subcellular fractions by immunoblotting as described above.

RT-PCR—cDNA was prepared by random hexamer priming, and 500 ng of total RNA as template was prepared from control or LEDGF/p75-deficient cell lines. Reactions were carried out with the ProStar Ultra HF RT-PCR system (Stratagene). Minus RT controls omitted only the reverse transcriptase. One-seventeenth of the total cDNA was used in a PCR reaction to amplify full-length HIV-1 IN with specific sense 5'-ATATGGATCCAATGTTTTAGATGG-3' and antisense 5'-ATATAC-CGGTGGGTCTCATCCTGTC-3' primers. Glyceraldehyde-3-phosphate dehydrogenase primers were used for the RNA loading control.

RESULTS

LEDGF/p75-deficient Cell Lines Express Lower Levels of Lentiviral Integrase Proteins despite Equivalent mRNA—Stable expression of IN proteins was used to optimize assessments of interactions with endogenous cellular proteins, analyze steady state IN levels, and allow tracking of IN through the cell cycle. Puromycin selection was used to stably express single monocistronic mRNAs that contain a lentiviral IN gene (HIV-1 or FIV IN) and a downstream (internal ribosome entry site-linked) puromycin resistance gene (see “Experimental Procedures”). The puromycin-stable transfectants were derived

² M. Vanegas, manuscript in preparation.

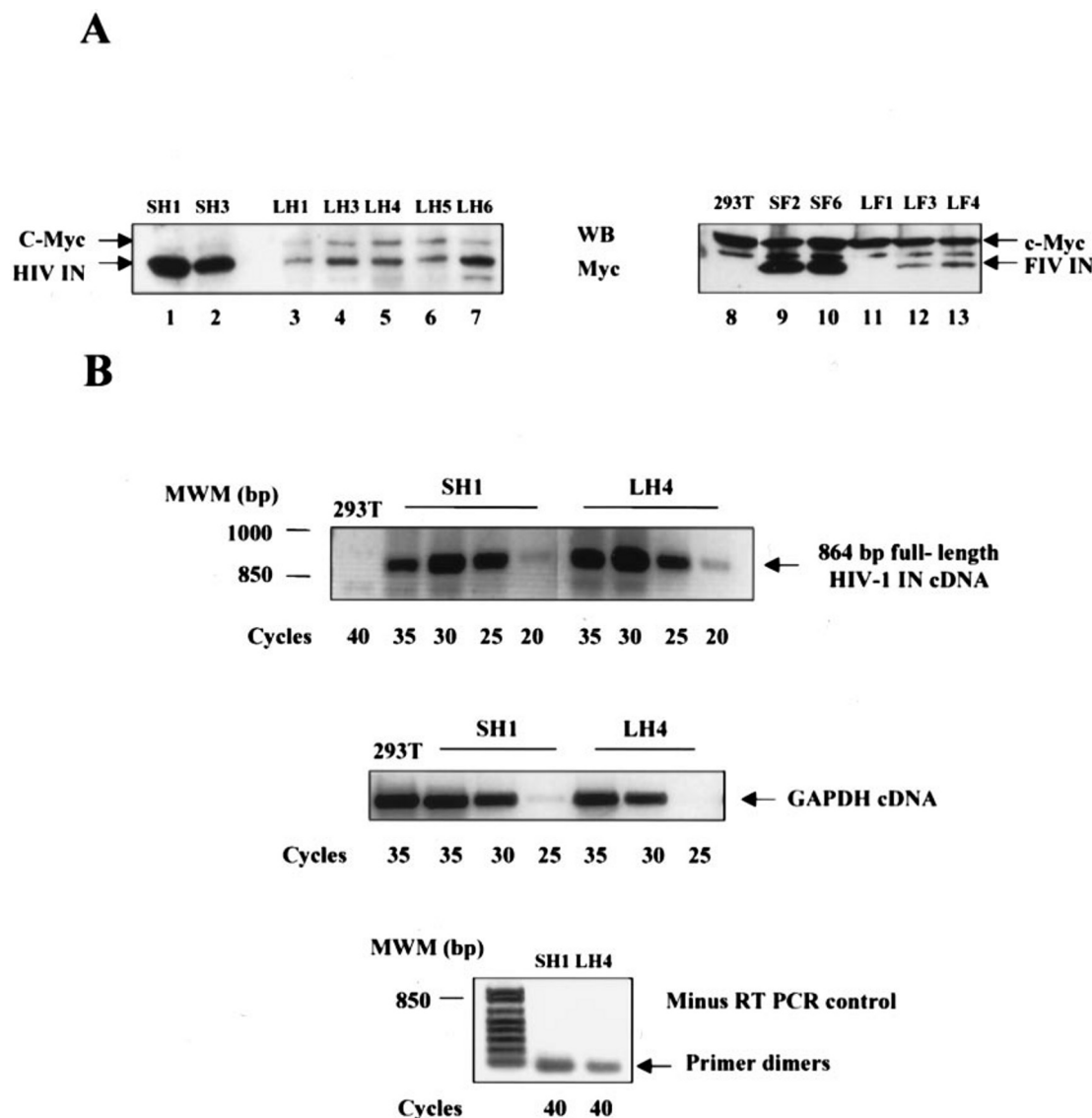


FIG. 1. Lentiviral integrase expression in LEDGF/p75-deficient cells. A, lentiviral IN protein expression was evaluated by Western blotting in L and S cell-derived clones that stably express Myc-tagged HIV-1 (LH and SH cells) or FIV (LF and SF cells) integrase proteins. Thus, the cell line nomenclature used is summarized as follows: the first letter (L or S) refers to the stably expressed shRNA (anti-LEDGF/p75 or scrambled control shRNA), and the second letter refers to the stably expressed lentiviral integrase (HIV-1 or FIV). A number following H or F identifies a particular single-cell clone derived from LH, SH, LF, or SF cells. An endogenous protein (upper arrows, probably a degradation fragment of endogenous c-Myc) is detected equivalently in all cells by the anti-Myc mAb. WB, Western blot. B, IN mRNA levels. RT-PCR was performed with primers that amplify the full-length cDNA. Minus RT controls are shown at bottom. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) verifies equal loading. MWM, molecular weight measurement.

from a cell line previously made severely LEDGF/p75-deficient by stable expression of shRNAs (si1340 or L cells (7)), thus generating LH and LF cells, respectively. They were also derived from the control line, which expresses a scrambled shRNA (siScram or S cells (7)), generating SH and SF cells, respectively. LH and LF cells expressed much lower steady state levels of HIV-1 and FIV IN proteins than did SH or SF cells (Fig. 1A). This LEDGF/p75 dependence was observed consistently both in the four polyclonal founder cell lines (data not shown) and in multiple randomly selected single-cell clones (Fig. 1A). All LH ($n = 5$) and LF ($n = 6$) cell clones had markedly lower steady state levels of the respective IN protein than did SH ($n = 9$) and SF ($n = 11$) cell clones. In addition, lower levels of HIV-1 IN were detected in L cells than in S cells when IN was expressed by transient transfection (data not shown).

Available data suggest the normal role of LEDGF/p75 is to act as a transcriptional coactivator (1). Therefore, one possibil-

ity to explain the lower steady state levels of IN proteins in LH and LF cells as compared with SH and SF cells was lower IN mRNA production. However IN mRNA levels were equivalent in LH and SH cells, even those most divergent in IN protein steady state levels (Fig. 1B), indicating a post-transcriptional mechanism. The equivalent IN mRNA levels are consistent with the way the cell lines were derived, with uniform selection pressure at 3 μ g/ml puromycin for the monocistronic mRNAs encoding both IN and puromycin resistance. Growth rates were also the same for LH and SH cells in the presence of the drug (data not shown).

HIV-1 and FIV INs Undergo Proteasomal Degradation and Are Ubiquitinated—Because degradation by the proteasome is an important post-transcriptional regulator of protein levels, we evaluated its role in the lower steady state IN levels in LEDGF/p75-deficient cells. Cells were treated with the proteasome inhibitor MG132 and evaluated by immunoblotting (Fig. 2, compare lanes 1–4 with lanes 5–8 and compare lanes 9–11

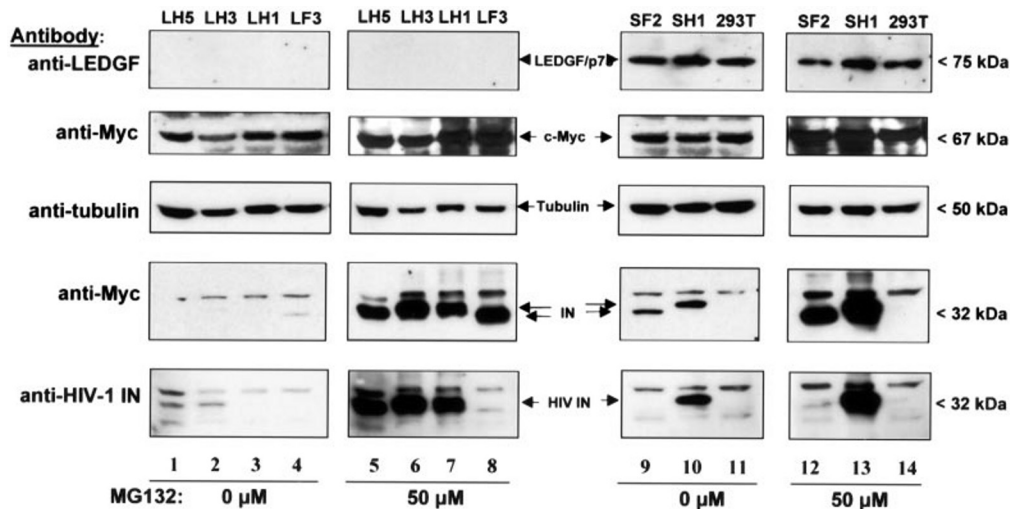


FIG. 2. **Proteasome inhibition enhances HIV-1 and FIV IN expression.** LH and SH clones were incubated in the absence or the presence of the proteasome inhibitor MG132 and evaluated by immunoblotting with four different antibodies. Both lentiviral INs are detected with the anti-Myc mAb (fourth panel row); HIV-1 IN was also evaluated with a specific rabbit antiserum (fifth panel row). LEDGF/p75 and tubulin were detected with specific monoclonal antibodies to assess LEDGF/p75 expression and loading, respectively.

with lanes 12–14). Results obtained in the absence of MG132 confirmed the data of Fig. 1A, with lower IN protein levels in LH and LF cells than in SH and SF cells (Fig. 2, lanes 1–4, 9, and 10). Note also that the LEDGF/p75 knock-down remained stringent in L cells through the selection for HIV-1 and FIV IN expression (Fig. 2, lanes 1–8 versus lanes 9–14). MG132 caused a marked increase in the levels of both lentiviral IN proteins. This increase could be detected in both L and S clones, but the magnitude was much larger in L clones, where both lentiviral IN proteins increased to WT from undetectable or barely detectable levels (Fig. 2, compare lanes 1–4 with lanes 5–8). c-Myc, a known target for proteasome degradation (43), was also more abundant in the MG132-treated cells, although the effect on IN proteins was larger (Fig. 2). Moreover, the levels of other host proteins such as tubulin and, more importantly, of LEDGF/p75, decreased slightly after proteasome inhibition (Fig. 2). These data indicate that HIV-1 IN levels are determined by proteasome degradation and that LEDGF/p75 regulates this process.

Proteasome targeting results from polyubiquitination. We transiently expressed HA-tagged ubiquitin (41) in SH and LH cell clones that stably express HIV-1 IN. 24 h after transfection cells were treated with MG132 for 18 h, and cellular lysates were reciprocally immunoprecipitated for HA-ubiquitin or IN followed by Western blotting for IN and HA-ubiquitin, respectively (Fig. 3, A and B). Higher molecular mass ubiquitinated forms (>37.1-kDa marker) of HIV-1 IN were specifically immunoprecipitated from both LH and SH cell clones by both approaches. Although SH1 cells express higher levels of IN than LH4 cells (Fig. 1A), ubiquitinated IN was more abundant in LH4 cells when ubiquitin-modified proteins were directly immunoprecipitated (Fig. 3A). When IN was immunoprecipitated, ubiquitinated IN was specifically detected in both types of cells (Fig. 3B).

Cytoplasmic HIV-1 IN Has a Shorter Cellular Half-life than Nuclear HIV-1 IN—HIV-1 IN is found exclusively in the cytoplasm of L cells when analyzed by immunofluorescence (7). In LEDGF/p75 WT cells, HIV-1 IN protein localizes overwhelmingly to nuclei, although some IN can be immunoprecipitated from the cytoplasmic fraction. Here, Western blotting of subcellular fractions from LH and SH clones was carried out and revealed that the majority of HIV-1 IN was found in the nuclear fraction of SH clones (Fig. 4, upper panel). In contrast, no IN was detected in nuclei of LH cells, further supporting our

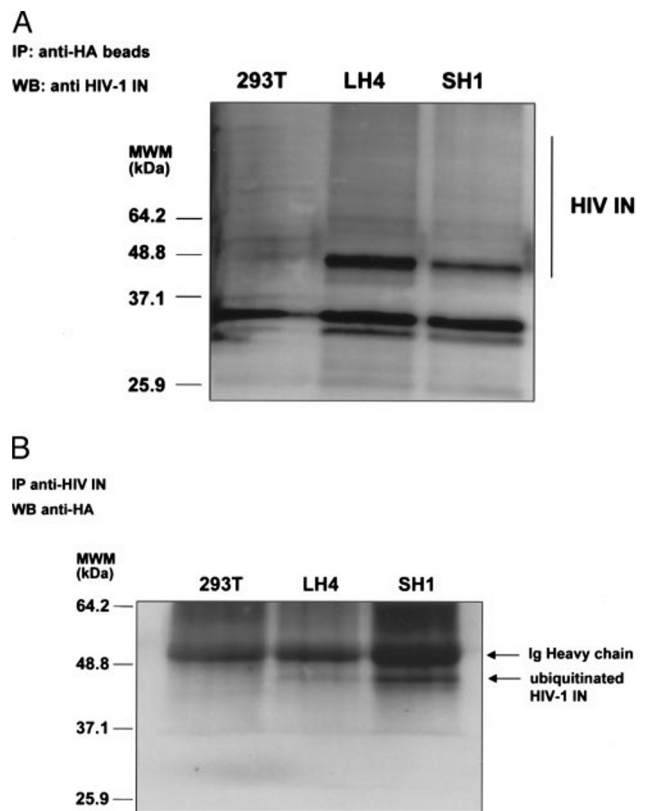


FIG. 3. **HIV-1 IN is targeted for ubiquitination.** LEDGF/p75-deficient (LH4) and LEDGF/p75 wild type (SH1) cells that stably express HIV-1 IN were transfected with pUbiquitin-HA (41). Cellular lysates were immunoprecipitated with anti-HA mAb (A) or with rabbit antibody to HIV-1 IN (B) and then evaluated by immunoblotting with rabbit anti-HIV-1 IN or with anti-HA mAb, respectively. No ubiquitinated IN bands were detected in immunoprecipitates from control 293T cells transiently transfected with pUbiquitin-HA or from LH4 and SH1 cells that were not transfected with pUbiquitin-HA (data not shown). IP, immunoprecipitate; WB, Western blot; MWM, molecular weight measurement.

model in which LEDGF/p75 is required for nuclear location of IN. Interestingly, however, although the total levels of HIV-1 IN differed markedly, the cytoplasmic IN pools in SH and LH cells were equivalent.

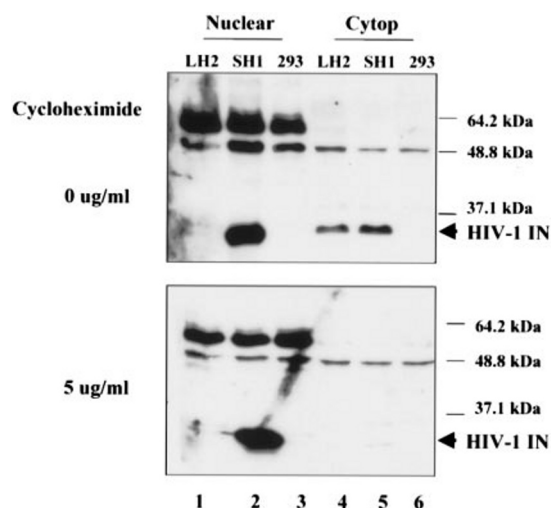


FIG. 4. Subcellular distribution and stability of HIV-1 IN. Detection of HIV-1 IN by Western blotting in nuclear and cytoplasmic (Cytop) fractions of LH and SH cell clones with or without treatment with cycloheximide. 293T cells were included as a control for specificity.

We therefore evaluated how subcellular distribution of HIV-1 IN influences its stability. Cells were incubated in the presence of cycloheximide for 30 min at 37° C to permit protein decay while preventing *de novo* synthesis and to allow, in SH cells, the nuclear accumulation of preformed cytoplasmic HIV-1 IN protein. Then cells were transferred to ice until analysis of HIV-1 IN by immunoblotting. As shown in Fig. 4 (lower panel), the nuclear pool of HIV-1 IN in SH cells was entirely stable, whereas the cytoplasmic pools in both SH and LH cells completely decayed.

The rapid loss of cytoplasmic IN in SH cells could be influenced by two processes, nuclear transport and cytoplasmic degradation. However, in LH cells only cytoplasmic degradation could explain the decay because nuclear IN was never detected in these cells (Fig. 4). These results indicated that cytoplasmic HIV-1 IN has a shorter half-life than nuclear HIV-1 IN.

LEDGF/p75 Rescues Lentiviral INs from Proteasome Degradation—To verify that LEDGF/p75 regulates proteasomal degradation of HIV-1 IN, we transiently expressed LEDGF/p75 in LH cells. To override the stable RNAi in LH cells, we modified the LEDGF/p75 cDNA to contain seven synonymous mutations within the 18-nucleotide target site of the shRNA constitutively present in LH cells (generating pLEDGFsiMut). pLEDGFsiMut-transfected LH cells exhibited a marked increase in the levels of IN proteins in all clones (Fig. 5A). The rescue effect was specific because the levels of other endogenous proteins such as c-Myc (Fig. 5A) or tubulin (not shown) were unchanged. In addition, the specificity of this effect was shown by transfection of LEDGF/p52, which does not interact with integrase (7) and is more active as a transcriptional coactivator (1). LEDGF/p52 was equally overexpressed, yet it had no effect on the levels of HIV-1 IN in LEDGF/p75-deficient or normal cells, whereas LEDGF/p75 again dramatically increased HIV-1 IN (Fig. 5B). Note also the complete concordance between IN and LEDGF/p75, whether the latter is endogenous (lanes 1, 2, 4, 5) or overexpressed (lanes 3 and 6); in addition, this figure confirms the much lower levels of endogenous p52 versus p75 (Fig. 5B). These results further exclude the possibility that the rescue of IN by LEDGF/p75 overexpression is a transcriptional effect.

IN Stabilization Requires Interaction with LEDGF/p75 but Not Nuclear Residence of Chromatin Tethering—Because the

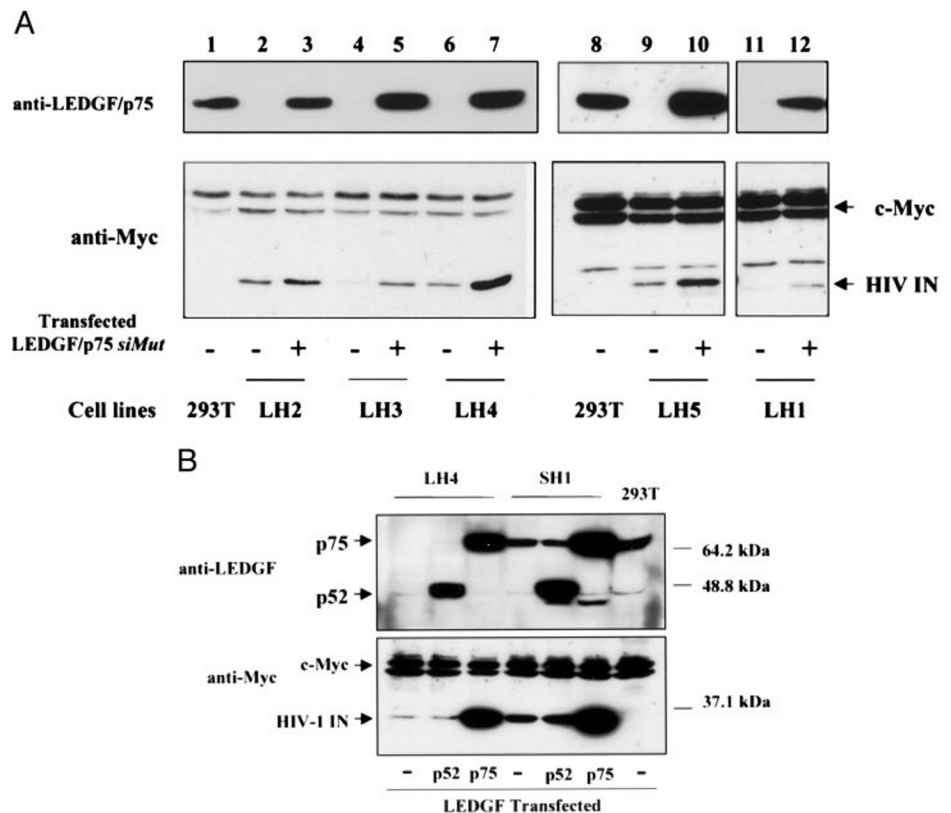
nuclear pool of IN is more stable than the cytoplasmic pool, and reintroduction of LEDGF/p75 causes nuclear relocation of IN, it was possible that the effect of LEDGF/p75 on HIV-1 IN stability is primarily a function of nuclear residence of the proteins. To determine whether nuclear location or tethering of IN to chromatin by LEDGF/p75 is necessary for preventing IN decay, we used a LEDGF/p75 nuclear localization mutant that is alanine-substituted for 4 contiguous basic amino acids in an N-terminal classical NLS (residues 149–152) and retains interaction with IN but is cytoplasmic.² To enable its expression in LH cells, this NLS mutation was constructed in the LEDGF/p75siMut background, generating pLEDGF/p75siMut.NLS⁻. Transient transfection of either pLEDGFsiMut or pLEDGFsiMut.NLS⁻ markedly increased the levels of HIV-1 IN in SH and LH cells with similar potency, confirming the data of Fig. 5A (Fig. 6A). However, increases in IN protein were independent of nuclear/chromatin localization, because the NLS mutant LEDGF/p75 and HIV-1 IN were found only in the cytoplasm of LH cells, where they exactly colocalized (Fig. 6B). In addition to increasing the levels of the endogenously expressed HIV-1 IN, the reintroduction of wild type LEDGF/p75 relocated IN completely to the nucleus, confirming that the cytoplasmic localization of IN in these cells is due specifically to lack of LEDGF/p75 expression (Fig. 6B).

To verify that the ability of LEDGF/p75 to stabilize IN requires the formation of a complex between these two proteins, LH and SH cells were transfected with pLEDGF/p75siMut.Δ340–417, which encodes LEDGFΔ340–417. This protein does not interact detectably with IN in immunofluorescence analyses because of a deletion of C-terminal residues 340–417.² The Δ340–417 mutant and wild type LEDGF/p75 were assessed by Western blotting (Fig. 6A). In contrast to the major increase induced by wild type LEDGF/p75, Δ340–417 only marginally increased HIV-1 IN expression levels in both SH and LH cells, indicating that HIV-1 IN stabilization by LEDGF/p75 requires the interaction of the proteins. In direct further support of this conclusion, the Δ340–417 protein and HIV-1 IN were found in different cellular compartments (Fig. 6C). The differences in the abilities of these LEDGF/p75 proteins to rescue the expression of HIV-1 IN in LH cells were not a result of differential expression levels, as these were equivalent in the transfected cells (Fig. 6A).

DISCUSSION

Specific RNAi knock-down of LEDGF/p75 is well tolerated by human cells, yet it produces a dramatic phenotype for lentiviral integrase proteins, which relocate from the nucleus, where they are tightly associated with chromatin, to an exclusively cytoplasmic location (7). In addition, the colocalization of endogenous LEDGF/p75 and HIV-1 IN remains complete throughout the cell cycle (7). Type C murine oncoretroviral integrase proteins do not interact with LEDGF/p75 and remain cytoplasmic in its presence and absence (7). Current published data support a role for LEDGF/p75 as a main chromatin “tethering factor” accounting for the nuclear localization of HIV-1 IN (7, 14, 15), a model that supplants earlier hypotheses for classical NLSs in this viral protein. Although other tethering factors may exist, LEDGF/p75 appears to be the most abundant because in LH and LF cells, which express moderate levels of IN proteins, integrase is found entirely in the cytoplasm by immunofluorescence and immunoblotting. The present work shows that the second function of LEDGF/p75 with regard to HIV-1 IN is to serve as the main determinant of its stability in cells. Without LEDGF/p75, HIV-1 (and FIV) IN turn out to be extremely unstable and accumulate to barely detectable levels. Proteasome degradation accounts for this instability. Moreover, the role of LEDGF/p75 in protecting integrase is independent of the locations of the proteins in the cell.

FIG. 5. LEDGF/p75 but not LEDGF/p52 overexpression increases endogenous HIV-1 IN levels in LEDGF/p75-deficient clones. **A**, detection of HIV-1 IN by Western blotting in LH cell clones stably expressing HIV-1 IN transiently transfected or not with a LEDGF/p75 expression plasmid. WT 293T cells were included as a control of specificity and to show endogenous levels of LEDGF/p75. **B**, comparison of LEDGF/p52 and LEDGF/p75 overexpression in LH4 and SH1 cells. The upper panel shows the transfected LEDGF proteins detected with an antibody specific for the N terminus, which is shared by both p52 and p75. Endogenous LEDGF proteins are also detected, although endogenous p75 is not found in stably knocked down LH4 cells. The lower panel shows IN expression in the same samples, detected with mAb to the Myc epitope tag at the C terminus of IN.



Our stable expression methods for LEDGF/p75 shRNAs and integrase proteins led to the primary observation that integrase protein levels are severely reduced in cells that lack LEDGF/p75. This expression pattern was highly consistent and observable in all clones derived from these stable cell lines under conditions that facilitated equivalent transcription. Accordingly IN mRNA levels did not correlate with steady state IN protein levels, whereas LEDGF/p75 levels did. Proteasome inhibition in LEDGF/p75-deficient cells resulted in full reversal of the IN protein deficit.

The restoration of IN expression by LEDGF/p75 overexpression in LH cells directly supports the conclusion for a specific protective role of LEDGF/p75, presumably acting autonomously. A similar effect, lower in magnitude, was also observed with overexpression in SH cells. Taken together, these data indicate that in normal cells LEDGF/p75 levels are the main limiting factor for HIV-1 IN expression. LEDGF/p75 transfection did not increase levels of other endogenous proteasome-targeted proteins such as c-Myc. Indeed, we show that unless proteasome inhibition is utilized, it is difficult to express and detect HIV-1 IN in the absence of LEDGF/p75, *i.e.* in L cells, even with maximal transient transfection methods and constructs that produce high level expression in LEDGF/p75 WT cells.

The subcellular localization experiments produced unambiguous striking results that fit the model of LEDGF/p75 action. Overexpression of LEDGF/p75 but not LEDGF/p52 in LH cells not only rescued stability but also induced relocation of IN from the cytoplasm to the nucleus. We considered the possibility that LEDGF/p75-mediated proteasome shielding could be due to this nuclear location *per se*. However, the proteasome is active in the nucleus as well as the cytoplasm (17–22). The possibility that the association of HIV-1 IN with chromatin or nuclear residence is required for protection from the proteasome was excluded when LEDGF/p75 mutants that lack the nuclear localization signal or the domain required for interac-

tion with IN were studied. Protection clearly did not require nuclear localization of the IN-LEDGF complex. Only LEDGF/p75 proteins that can interact with HIV-1 IN can stabilize it. HIV-1 IN was ubiquitinated in both LH and SH cells, and the levels of Ub-modified protein correlated inversely with the total steady state IN levels when ubiquitinated proteins were directly immunoprecipitated. Lentiviral INs are exclusively cytoplasmic in LEDGF/p75-deficient cells, whereas they are predominantly nuclear in WT cells. The cytoplasmic HIV-1 IN pool has a shorter half-life than the nuclear pool (Fig. 4). Our results support and extend previous reports that MG132 treatment of cells increases expression of IN (33) and N-terminal ubiquitin-IN fusions (34).

Exactly how LEDGF/p75 protects IN from degradation deserves investigation for its interest to HIV-1 and proteasome cell biology. The protection IN receives from LEDGF/p75 is a third instance of an HIV-1 protein manipulating the proteasome system, one in which proteasome degradation of an HIV-1 protein is specifically inhibited. Vif and Vpu both promote degradation of cellular factors by the proteasome (35–39). The requirement of interaction between LEDGF/p75 and IN for IN stabilization and the lack of effects on other cellular proteins indicate a specific molecular masking rather than a general effect on the proteasome pathway. That pathway begins with attachment of ubiquitin in a series of enzymatic reactions (23, 24). After ATP activation by the ubiquitin-activating enzyme E1, the molecule is transferred onto the carrier protein E2. In the third step, ubiquitin ligase (E3) covalently ligates ubiquitin to lysine residues of the target protein. Our data are compatible with a possibility that LEDGF/p75 may protect IN from engagement of the E3 ligase itself. We note that the proteasome-shielding effect mediated by LEDGF/p75 is similar to three precedents in other systems. RUNX1/AML1, a transcription factor essential for hematopoiesis, is degraded continuously by the ubiquitin-proteasome pathway (44). Heterodimerization between RUNX1/AML1 and the transcription factor PEbP2 β

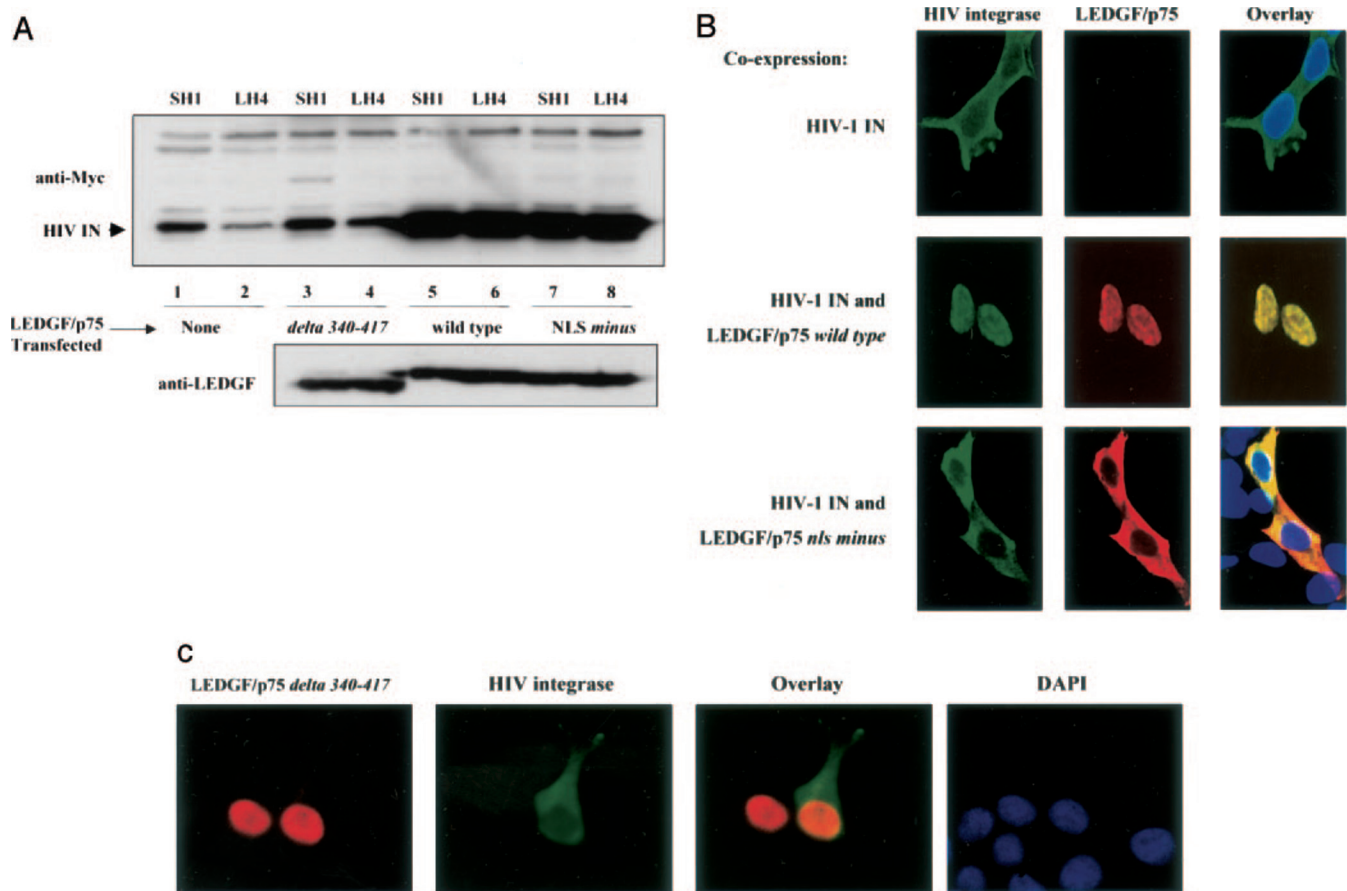


FIG. 6. HIV-1 IN is stabilized upon interaction with LEDGF/p75 independent of its subcellular localization. *A*, detection of HIV-1 IN by Western blotting in LEDGF/p75-deficient cells transiently transfected with wild type LEDGF/p75 (pLEDGF/p75siMut), or mutants lacking the nuclear localization signal (pLEDGF/p75siMut.NLS⁻), or a region required for interaction with IN (pLEDGF/p75siMut.Δ340–417). *B* and *C*, confocal immunofluorescence showing subcellular localization in L cells of HIV-1 IN (green) and NLS-mutant LEDGF/p75 (red) (*B*) or HIV-1 IN (green) and LEDGF/p75Δ340–417 (red) (*C*). No endogenous LEDGF/p75 is detectable in L cells because of the stable RNAi; the RNAi is overridden in transfections by using the siMut forms of all LEDGF/p75 expression plasmids. DAPI, 4',6-diamidino-2-phenylindole.

abrogates ubiquitinylation of RUNX1/AML1 and markedly inhibits the proteolysis (44). PEBP2β-deficient cells have barely detectable RUNX1/AML1, a situation that parallels the barely detectable IN levels in L cells. Similarly, hRad1, one of three Rad family proteins that form a heterotrimeric complex involved in DNA integrity checkpoint pathways, can associate with one of the two others (hHus1) in the absence of the third (hRad9), thus protecting the otherwise very unstable hHus1 from ubiquitination and proteasome degradation in the cytoplasm (45). Genotoxic stress appears to induce Rad1 expression and stabilize hHus1 (45). A third example is formed by two subunits of the yeast RNA polymerase II C-terminal domain kinase complex, the cyclin Ctk2 and the regulatory protein Ctk3, which physically interact to protect each other from degradation by the ubiquitin proteasome (46).

The part this provocative protein interaction plays in the HIV-1 life cycle remains to be determined. Whether LEDGF/p75 serves as an autonomous direct linker between chromosomal DNA and integrase proteins or whether the observed tethering phenomenon involves a more complex protein ensemble is not certain at present. It is now known that the interaction is not confined to situations where IN is expressed in the absence of other viral proteins because LEDGF/p75 is a component of HIV-1 preintegration complexes isolated from infected cells (7). However, LEDGF/p75 has not so far been shown to be essential for viral replication because severely LEDGF/p75-deficient cell lines support HIV-1 replication (7). A requirement that is met by very low levels of LEDGF/p75 has

not been ruled out, however. A different role in a recently discovered clinically relevant phenomenon (the bias of HIV-1 integration toward actively transcribed genes (40, 47, 48)) has been hypothesized (7) and is under investigation. Because LEDGF/p75 is a chromatin-associated and abundant transcriptional coactivator, and it has been reported to augment integrase *in vitro* strand transfer activity (14), there is also a need to determine whether and how LEDGF/p75 participates in integrase catalysis as it occurs in cells.

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