2006-07-01

Modest but reproducible inhibition of human immunodeficiency virus type 1 infection in macrophages following LEDGFp75 silencing

Steven P. Zielske
University of Massachusetts Medical School

Et al.

JOURNAL OF VIROLOGY, July 2006, p. 7275–7280 Vol. 80, No. 14
0022-538X/06/$08.00+0 doi:10.1128/JVI.02470-05

Modest but Reproducible Inhibition of Human Immunodeficiency Virus Type 1 Infection in Macrophages following LEDGFp75 Silencing

Steven P. Zielske† and Mario Stevenson*

Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Received 23 November 2005/Accepted 3 May 2006

LEDGFp75 is a cellular protein which binds human immunodeficiency virus type 1 (HIV-1) integrase with high specificity and affinity but whose function in infection has not been defined. We infected LEDGFp75-deficient primary macrophages with wild-type HIV in order to assess potential infection phenotypes which would provide clues to LEDGFp75 function. Silencing of LEDGFp75 by 70 to 80% resulted in an average of 53% reduced infection of macrophages by HIV. Analysis of infection intermediates showed that integration, but not two-long-terminal-repeat (2LTR) circles or late cDNAs, was reduced up to 74% in LEDGFp75-deficient macrophages. Therefore, LEDGFp75 has a modest involvement in HIV-1 integration in macrophages.

Human immunodeficiency virus type 1 (HIV-1) cDNA is integrated into cellular DNA through a biochemically described process dependent on the catalytic activity of viral integrase protein. The preintegration complex (PIC) consists of a multiprotein complex containing viral and cellular proteins. Several cellular proteins have been found to interact with integrase in vitro and stimulate the in vitro integration reaction. These proteins include BAF, HMGA, INI1, and LEDGFp75 (lens epithelium-derived growth factor p75) (3, 6–8).

LEDGF exists in two major splice forms, which result in 52- and 75-kDa proteins (13). While the 75-kDa form interacts with integrase, the 52-kDa form lacks the integrase-binding region (11). LEDGFp75 is a nuclear protein which functions as a transcription factor for LEDGF-responsive genes (12). Initial work using RNA interference (RNAi) to knock down LEDGFp75 in cell lines caused exogenously expressed integrase to be defective in nuclear accumulation and chromosomal tethering (1, 9, 11, 14). Curiously, disagreement exists about whether an infection defect can be attributed to LEDGFp75-deficient cells (5, 9). This may be due to the fact that these studies utilized cell lines as experimental models of HIV infection rather than natural targets or integrase mutants which potentially have pleiotropic defects (10).

In the work described here, we investigated HIV infection of human primary macrophages by replication-competent HIV-1 following RNAi of LEDGFp75. We found modest but reproducible inhibition of macrophage infection in approximate proportion to the degree of LEDGFp75 knockdown. We localized this defect to occur after nuclear entry at the step of integration. Nuclear import is unaffected. Our data support a hypothesis in which LEDGFp75 increases in vivo integration efficiency by favoring the interaction of the PIC with genomic DNA.

Viral cDNA coimmunoprecipitates with LEDGFp75 in macrophages. Previous work has shown that LEDGFp75 binds exogenously expressed integrase protein (3). Llano et al. (9) showed not just that LEDGFp75 is a binding partner of integrase but also that association occurs in the context of functional HIV vector PICs in 293T cells. We therefore set out to determine whether LEDGFp75 bound viral cDNA-containing complexes in primary macrophages infected with wild-type HIV by looking for viral cDNA in anti-LEDGFp75 immunoprecipitates.

To accomplish this, we used macrophages differentiated from elutriated monocytes obtained from healthy donors. Macrophages were infected with vesicular stomatitis virus G (VSV-G)-pseudotyped HIV^{LAI} (produced in 293T cells), and cell extracts were collected in radioimmunoprecipitation assay buffer 12 h postinfection. LEDGF was immunoprecipitated using anti-LEDGF antibody (Becton Dickinson), and the final pellet was subjected to PCR using primers in *gag* and the LTR (W primer, 5′-GGGAGCTCTCTGGCTAACT-3′; BB primer, 5′-GGATTAACTGCGAATCGTTC-3′) to detect the presence of viral cDNA. Three controls were used: an uninfected macrophage sample, an infected sample immunoprecipitated with anti-LIG1 (Abcam), and an uninfected sample spiked with the infectious molecular clone plasmid used for HIV preparation.

HIV viral cDNA was detected in the LEDGF precipitate from the HIV-infected sample, while no signal was observed in the uninfected sample, the infected sample immunoprecipitated with anti-LIG1 antibody, or the plasmid spike control (Fig. 1). No signal with the plasmid spike indicates that a positive signal is not due to DNA carryover and that LEDGFp75 does not bind directly to viral cDNA under these conditions. These data show that LEDGFp75 associates with the PIC of wild-type HIV in human primary macrophages.

* Corresponding author. Mailing address: 373 Plantation St., Suite 319, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605. Phone: (508) 856-3645. Fax: (508) 856-4075. E-mail: Mario.Stevenson@umassmed.edu.

† Present address: Department of Radiation Oncology, University of Michigan, Ann Arbor, MI 48109.

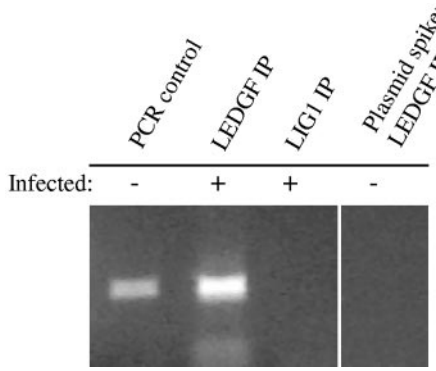

FIG. 1. Coimmunoprecipitation of viral cDNA. HIV-infected macrophage extracts were immunoprecipitated with anti-LEDGF or anti-LIG1 (control) antibody, followed by PCR analysis of pellets for the presence of viral cDNA. "PCR control" indicates PCR of an HIV clone plasmid. The last lane was uninfected macrophage extracts spiked with 90 ng HIV clone plasmid before LEDGF immunoprecipitation. Viral cDNA could be detected in infected extracts immunoprecipitated with anti-LEDGF antibody but not anti-LIG1 antibody, indicating LEDGF association with a viral cDNA complex in wild-type-infected primary macrophages. The signal was not due to carryover plasmid, since an irrelevant antibody produced no signal.

Impaired infection of macrophages deficient in LEDGFp75. Although LEDGFp75 binds to integrase, no significant infection defect has been observed in LEDGFp75-deficient cell lines by us (data not shown) and others (9). However, this question has not been investigated in primary cells with natural HIV infection. We thus chose human macrophages as a natural HIV target for investigation of the effect of LEDGFp75 deficiency on infection.

LEDGFp75 expression was silenced in macrophages by transfection of LEDGFp75-specific small interfering RNA (siRNA) (5′-AGACAGCATGAGGAAGCGATT-3′; Ambion) once per day for 2 days as previously described (15). A nonspecific siRNA sequence (NS; 5′-TTCTCCGAACGTGTCACGTTT-3′) was transfected into cells as a control. The efficiency of LEDGFp75 knockdown was measured by quantitative RT-PCR of LEDGFp75 mRNA (primer LEDGF-1460F, 5′-CAGGTCACAATGCAACAAGC-3′; primer LEDGF-1671R, 5′-TTCGCTTCCTCATGCTGTCT-3′) and normalized to LIG3 mRNA (primer LIG3up, 5′-GGCTGGGAAGAGCTGGAAGATAAT-3′; primer LIG3dn, 5′-TGATCTGGGTCTTCGTGTTGTAGC-3′) and Western blotting. RT-PCR revealed a 70 to 80% reduction of LEDGFp75 mRNA for up to 3 days posttransfection, which roughly corresponds to the reduction in protein levels observed by Western blotting (Fig. 2A).

LEDGFp75 or NS control siRNA-transfected macrophages were infected with HIV^{NL4-3}/GFP, carrying a green fluorescent protein (GFP) marker gene in place of *nef*, on the day following the second siRNA transfection. Since NL4-3 is T tropic, these infections are single round. After 4 days, macrophages were harvested and subjected to flow cytometry in order to quantify the proportion of infected cells according to GFP fluorescence (Fig. 2B). We found that macrophages undergoing LEDGFp75 silencing consistently exhibited decreased susceptibility to productive HIV infection compared to control cells, averaging 53% fewer HIV-infected, LEDGFp75-deficient macrophages (Fig. 2C; $P = 0.04$, $n = 5$). We achieved an average infection of 9.0% of NS control macrophages compared to 4.4% of LEDGFp75-deficient macrophages. Accordingly, virus output from LEDGFp75-deficient cultures 6 days postinfection was reduced approximately 67% (data not shown). LEDGFp75 silencing did not affect viral gene expression, since GFP mean fluorescence intensity (MFI) was unchanged in infected cells, indicating that LEDGFp75 acted early in infection (Fig. 2D; $P = 0.10$, $n = 3$).

Decreased infection was not a consequence of cell death due to LEDGFp75 knockdown. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme released from the cell early upon damage to the cell membrane and death. To assess cell toxicity due to LEDGFp75 RNAi, we measured LDH release from siRNA-transfected macrophages 4 to 6 days posttransfection (Fig. 2D). We found no significant toxicity associated with LEDGFp75 knockdown, suggesting that decreased HIV infection of macrophages cannot be explained by toxic effects of LEDGFp75 deficiency or siRNA transfection.

It has been reported by Cherepanov et al. (2) that a motif present in the integration binding domain of LEDGFp75 is also present in other proteins. One of these, HRP-2, encoded by the HDGF2 gene, was shown to bind HIV integrase and stimulate integration in vitro. We considered that the residual infection observed following LEDGFp75 knockdown could be due to HRP-2 activity. Our preliminary results show that infection of macrophages in which HDGF2 has been silenced does not result in decreased infection. Neither is there a greater effect when HDGF2 is silenced together with LEDGFp75 (data not shown).

Overall, these data show that LEDGFp75 plays an important positive role in HIV infection of macrophages.

Impaired macrophage infection is the result of reduced HIV integration. The point at which LEDGFp75 activity is important for HIV infection of macrophages was investigated by measurement of the accumulation of viral cDNA intermediates and integration following LEDGFp75 knockdown and HIV infection. LEDGFp75 was silenced by RNAi, and the day following transfection, macrophages were infected with VSV-G-pseudotyped HIV^{LAI}. These infections were single round, as LAI cannot infect macrophages without VSV-G pseudotyping. DNA was prepared from macrophages 1 to 5 days postinfection using DNAzol, and viral cDNA intermediates (2LTR and late cDNAs) were quantified by real-time PCR, normalizing to CCR5 gene copies, as previously described (15).

Integrated provirus was quantified using real-time, single-step Alu-PCR. In this approach, PCR amplicons were directly quantified using primers in *gag* and genomic Alu repeats (*gag*, W primer [above]; AluR, 5′-TGCTGGGATTACAGGCGTGAG-3′) and a TaqMan probe complementary to viral sequence (5′-AGCCTCAATAAAGCTTGCCTTGAGTGC-3′). Values are relative, as was the DNA standard, thus rendering inequities in amplicon amplification efficiencies equivalent between standards and unknowns. Integrated provirus could be detected after 25 to 35 cycles, was reproducible among replicates, and generated standard curves with R^2 values near 1.0 and slopes near 3.3 without resorting to nested PCR (Fig. 3A). In Fig. 3B, we show the results of three experiments where completion of reverse transcription (late cDNAs), nuclear import

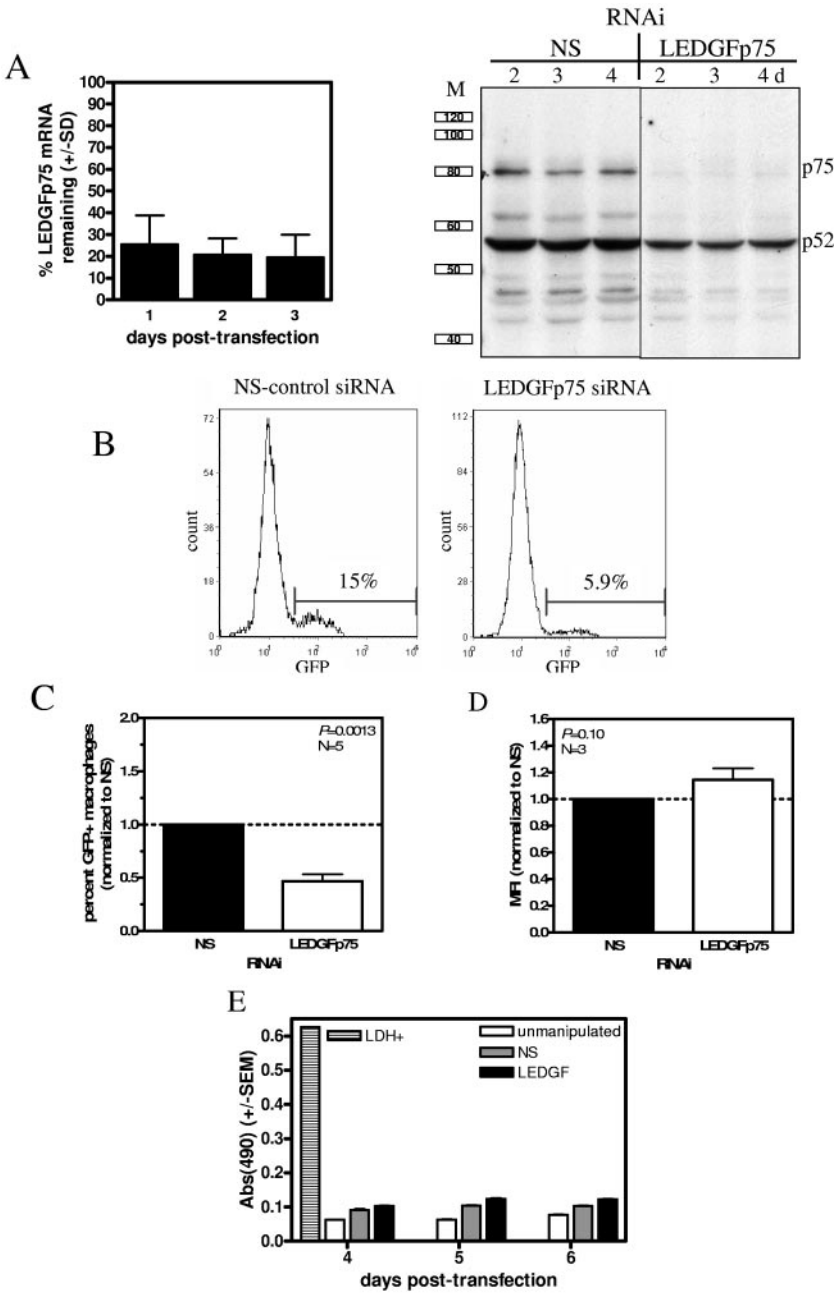

FIG. 2. Infection of LEDGFp75-deficient macrophages. A. Analysis of LEDGFp75 RNAi by RT-PCR and Western blotting. LEDGFp75 mRNA was quantified by real time RT-PCR, normalized to LIG3 mRNA, and compared to NS control siRNA-transfected cells. LEDGFp75 mRNA levels were decreased 70 to 80%. The second panel shows a representative Western blot for LEDGF showing LEDGFp75 protein knockdown at 2 to 4 days posttransfection. B. NS control or LEDGFp75-silenced macrophages were infected with HIV^{NL4-3}/GFP, and the proportion of infected cells was measured by flow cytometry. A representative flow cytometry histogram shows that macrophages deficient in LEDGFp75 displayed reduced susceptibility to HIV infection. C. An average of five experiments shows that reduced infection of LEDGFp75-deficient macrophages was consistent and significantly lower than infection of NS control cells ($P = 0.0013$). D. Infected macrophage cultures were subjected to flow cytometry, and the MFI of GFP^+ cells was measured. Values for LEDGFp75-deficient cells were normalized to NS control cells. Data indicate that the infection defect observed is due to LEDGFp75 acting during early events in the viral life cycle. E. Potential cell toxicity due to LEDGFp75 deficiency or RNAi was assessed by measurement of LDH activity in culture supernatants following siRNA transfection. Only marginal increases in LDH activity were observed in siRNA-transfected cultures compared to unmanipulated cultures. LDH+, LDH^+ control from the assay kit.

(2LTR circles), and integration (Alu-PCR) were measured and compared to results for NS control cells.

Accumulation of late cDNAs was similar between LEDGFp75-deficient and NS controls cells (Fig. 3B and C), indicating that LEDGFp75 does not influence reverse transcription in macrophages. Kinetic analysis of 2LTR circle formation, a measurement of nuclear import, showed an increased over the period analyzed, in one case reaching a plateau by day 5 (ex-

periment 3). No significant decrease in 2LTR circle formation was observed in macrophages undergoing LEDGFp75 silencing, compared to NS control macrophages (Fig. 3B and C). These data indicate that LEDGFp75 does not influence translocation of the PIC to the nuclear compartment and that decreased infection in LEDGFp75-deficient macrophages is not due to decreased PIC nuclear import.

In following the progress of integration over a 5-day period, we found that macrophages deficient in LEDGFp75 consistently exhibited reduced integration by 5 days postinfection (Fig. 3B). In contrast, NS control cells generally continued to accumulate integrated provirus up to 5 days postinfection, the duration of analysis. The defect in integration was significant at 5 days postinfection ($P = 0.004$), with an average reduction compared to NS control cells of 74% (Fig. 3C). Decreased infection of LEDGFp75-deficient macrophages (Fig. 2) was therefore accompanied by a defect in integration, thus supporting a role for LEDGFp75 in this process.

In this study, we investigated the role of LEDGFp75 in HIV infection of macrophages. We found LEDGFp75 associated with a complex containing viral cDNA in infected macrophages, probably through interaction with integrase. Using RNAi technology, knockdown of LEDGFp75 resulted in a modest decrease in HIV infection of macrophages, roughly corresponding to the degree of LEDGFp75 knockdown. In preliminary work, HRP-2, another integrase binding protein, did not compensate for residual LEDGFp75 protein or result in decreased HIV infection upon its silencing (data not shown). Remaining macrophages infection may be due to incomplete LEDGFp75 silencing. Analysis of the part of the viral life cycle whereby LEDGFp75 functioned localized the activity to integration. Knockdown of LEDGFp75 had no effect on production of complete reverse transcripts or 2LTR circles; however, LEDGFp75-deficient macrophages displayed impaired, but not the absence of, integration, as measured by real-time PCR.

In other studies, investigators were unable to find a defect in HIV infection or the expression of a reporter gene incorporated into the virus in cell lines rendered stably deficient in LEDGFp75 (9). Likewise, we were unable to observe more than a slight effect on infection of HeLa cells (data not shown). The greater efficiency of infection and metabolic activity of cells lines than macrophages may negate the need for LEDGFp75. Second, it cannot be excluded that another protein present in various cell lines but not macrophages can compensate for the loss of LEDGFp75. Emiliani et al. (5) reported that integrase mutants unable to bind LEDGFp75 are defective for replication in cell lines. Integrase mutants are often plagued by unrelated pleiotropic defects (10). Nevertheless, our data using RNAi support their conclusions and extend them to primary cell targets of natural HIV infection.

The specificity and strength of the LEDGFp75-integrase interaction as determined by crystal structure analysis suggest an important role for LEDGFp75 in HIV biology (4). Initial work focused on potential nuclear import functions, since the mechanism of HIV PIC nuclear import is largely unknown. However, this hypothesis has not stood up to experimental results as described here, in macrophages, or in the work of others (9).

LEDGFp75 has been described as 'tethering' the PIC to genomic DNA (1, 5, 11, 14). We report impaired integration in LEDGFp75-silenced macrophages, a phenotype which is consistent with LEDGFp75 functioning in this manner. LEDGFp75 could also associate with other protein components of the PIC whereby loss of LEDGFp75 results in altered assembly/disassembly or stability of a complex which functions to enhance alignment of the PIC on target DNA.

In conclusion, we show that LEDGFp75 is able to bind to viral cDNA-containing complexes in macrophages. Silencing of LEDGFp75 expression results in a modest reduction in macrophage infection as a result of a defect in integration, while nuclear import and reverse transcription are unchanged. Overall, these data point to LEDGFp75 playing an

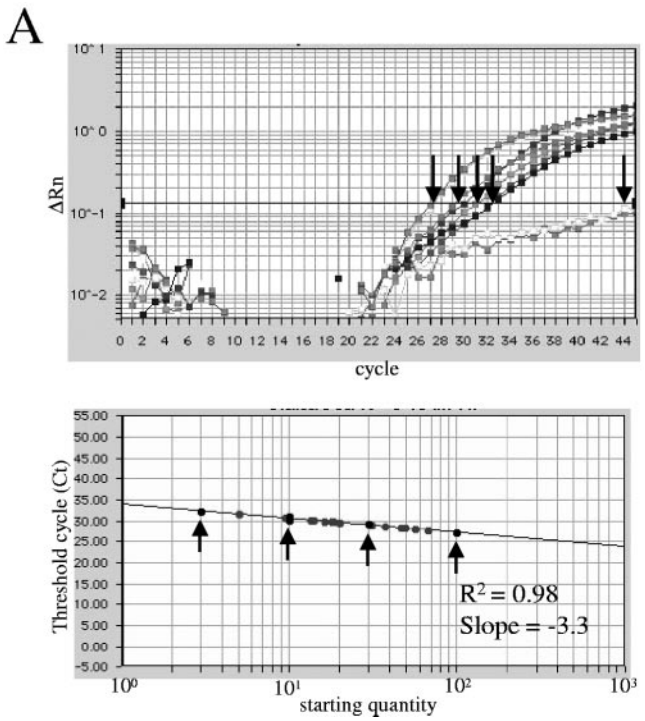

FIG. 3. Analysis of HIV life cycle intermediates. A. Viral integrations per cell were directly quantified using real-time PCR. Sensitivity was sufficient to detect integration without using nested PCR. The top panel shows a representative amplification plot of duplicate samples used for standard curve generation. These samples were derived from previously infected macrophage extracts which were serially diluted to establish the shown range. Arrows show the crossing point for each pair. The two samples not amplified are no-template controls. Curves for duplicates frequently overlap. The bottom panel shows the resulting standard curve. Arrows mark locations of standards. Other points are unknown samples. B. Late cDNAs, 2LTRs, and integrations were followed for 5 days following HIV infection of NS control or LEDGFp75-silenced macrophages. In experiments 2 and 3, an infected macrophage culture was treated with nevirapine as a negative control. Reverse transcription and nuclear import were largely normal in LEDGFp75-deficient cells, while integration was reduced, particularly at later time points. The 3-day time point for LEDGF in experiment 3 appears to be an outlier and possibly represents a technical error. C. LEDGFp75 data for each experiment were divided by the values for their corresponding NS controls to achieve normalized values and then averaged, to show that only integration was affected in LEDGFp75-deficient cells, reaching significance by 5 days postinfection ($P = 0.0044$).

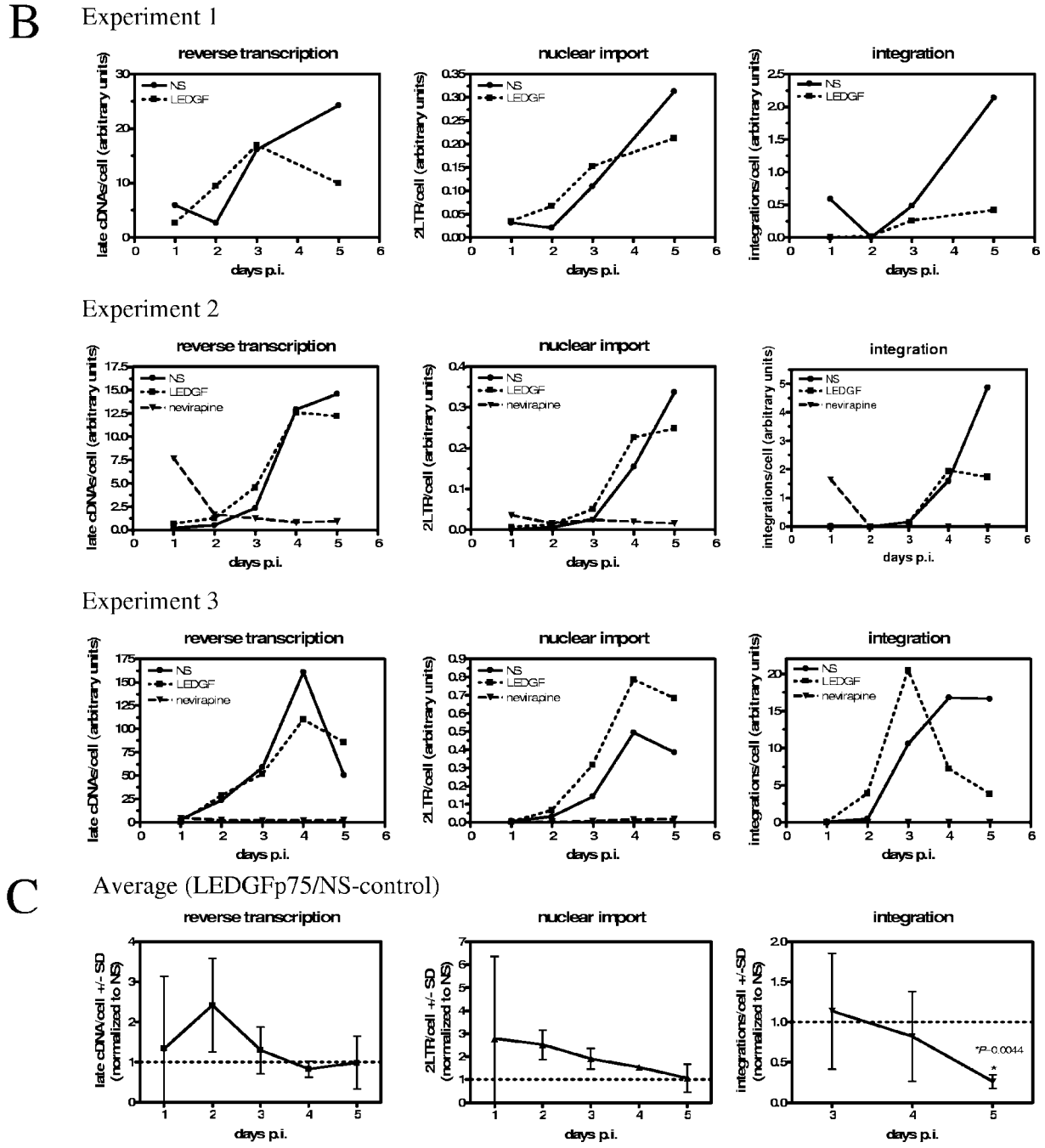

FIG. 3—*Continued.*

important role during HIV integration into macrophages and lay the groundwork for more targeted studies in the future.

We thank M. Sharkey and J. M. Jacque (University of Massachusetts) for helpful discussions.

This work was supported by NIH grants AI32890 and AI37475 and the University of Massachusetts Center for AIDS Research (AI042845). S.P.Z. is supported by a Kirschstein National Research Service Award (T32AI07349).

REFERENCES

1. **Busschots, K., J. Vercammen, S. Emiliani, R. Benarous, Y. Engelborghs, F. Christ, and Z. Debyser.** 2005. The interaction of LEDGF/p75 with integrase is lentivirus-specific and promotes DNA binding. J. Biol. Chem. **280:**17841–17847.
2. **Cherepanov, P., E. Devroe, P. A. Silver, and A. Engelman.** 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.
3. **Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. Van Beeumen, Y. Engelborghs, E. De Clercq, and Z. Debyser.** 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J. Biol. Chem. **278:**372–381.

4. **Cherepanov, P., Z. Y. Sun, S. Rahman, G. Maertens, G. Wagner, and A. Engelman.** 2005. Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75. Nat. Struct. Mol. Biol. **12:**526–532.
5. **Emiliani, S., A. Mousnier, K. Busschots, M. Maroun, B. Van Maele, D. Tempe, L. Vandekerckhove, F. Moisant, L. Ben-Slama, M. Witvrouw, F. Christ, J. C. Rain, C. Dargemont, Z. Debyser, and R. Benarous.** 2005. Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. J. Biol. Chem. **280:**25517–25523.
6. **Hindmarsh, P., T. Ridky, R. Reeves, M. Andrake, A. M. Skalka, and J. Leis.** 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.
7. **Kalpana, G. V., S. Marmon, W. Wang, G. R. Crabtree, and S. P. Goff.** 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science **266:**2002–2006.
8. **Lin, C. W., and A. Engelman.** 2003. The barrier-to-autointegration factor is a component of functional human immunodeficiency virus type 1 preintegration complexes. J. Virol. **77:**5030–5036.
9. **Llano, M., M. Vanegas, O. Fregoso, D. Saenz, S. Chung, M. Peretz, and E. M. Poeschla.** 2004. LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. J. Virol. **78:**9524–9537.
10. **Lu, R., A. Limon, E. Devroe, P. A. Silver, P. Cherepanov, and A. Engelman.** 2004. Class II integrase mutants with changes in putative nuclear localization signals are primarily blocked at a postnuclear entry step of human immunodeficiency virus type 1 replication. J. Virol. **78:**12735–12746.
11. **Maertens, G., P. Cherepanov, W. Pluymers, K. Busschots, E. De Clercq, Z. Debyser, and Y. Engelborghs.** 2003. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J. Biol. Chem. **278:**33528–33539.
12. **Sharma, P., D. P. Singh, N. Fatma, L. T. Chylack, Jr., and T. Shinohara.** 2000. Activation of LEDGF gene by thermal-and oxidative-stresses. Biochem. Biophys. Res. Commun. **276:**1320–1324.
13. **Singh, D. P., A. Kimura, L. T. Chylack, Jr., and T. Shinohara.** 2000. Lens epithelium-derived growth factor (LEDGF/p75) and p52 are derived from a single gene by alternative splicing. Gene **242:**265–273.
14. **Vanegas, M., M. Llano, S. Delgado, D. Thompson, M. Peretz, and E. Poeschla.** 2005. Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering. J. Cell Sci. **118:**1733–1743.
15. **Zielske, S. P., and M. Stevenson.** 2005. Importin 7 may be dispensable for human immunodeficiency virus type 1 and simian immunodeficiency virus infection of primary macrophages. J. Virol. **79:**11541–11546.