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ARTICLE

Efficient transduction of LEDGF/p75 mutant cells by complementary gain-of-function HIV-1 integrase mutant viruses

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Controlling the specificity of retroviral DNA integration could improve the safety of gene therapy vectors, and fusions of heterologous chromatin binding modules to the integrase (IN)–binding domain from the lentiviral integration host cofactor lens epithelium–derived growth factor (LEDGF)/p75 are a promising retargeting strategy. We previously proposed the utility of IN mutant lentiviral vectors that are selectively activated by complementary LEDGF/p75 variants, and our initial modifications in human immunodeficiency virus type 1 IN and LEDGF/p75 supported about 13% of wild-type vector transduction activity. Here we describe the selection and characterization of the K42E gain-of-function mutation in IN, which greatly improves the efficiency of this system. Both K42E and initial reverse-charge mutations in IN negatively affected reverse transcription and integration, yet when combined together boosted viral transduction efficiency to ~75% of the wild-type vector in a manner dependent on a complementary LEDGF/p75 variant. Although the K42E mutation conferred functional gains to IN mutant viral reverse transcription and integration, only the integration boost depended on the engineered LEDGF/p75 mutant. We conclude that the specificity of lentiviral retargeting strategies based on heterologous LEDGF/p75 fusion proteins will benefit from our optimized system that utilizes the unique complementation properties of reverse-charge IN mutant viral and LEDGF/p75 host proteins.

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INTRODUCTION

An obligate step in the retroviral replication cycle is the integration of the viral reverse transcript into a chromosome of the infected cell, a trait that makes retrovirus-based vectors particularly useful for gene therapy applications.[1] However, the benefits of efficient and stable gene delivery by retroviral vectors may be outweighed by risk of genotoxic effects, which include mutagenesis from vector insertion in the vicinity of proto-oncogenes. The recent successes and misfortunes of gene therapy trials call out for technologies to exert control over the specificity of retroviral DNA integration.[2]

Control of integration specificity has been approached through the modification of key viral and host cell proteins. Integration is catalyzed by the viral integrase (IN), which enters the cell as a virion component and associates with the viral DNA as it mobilizes to a suitable chromosomal acceptor site for integration.[3] Heterologous polydactyl zinc finger proteins fused to Moloney murine leukemia virus or human immunodeficiency virus type 1 (HIV-1) IN can alter integration specificity, though associated 10- to 10⁴-fold losses in vector titer may limit their practical application.[4,5] The family Retroviridae comprises seven genera (α through ε, spuma, and lenti), and the different viruses select for different chromatin features during integration. As examples, γ-retroviruses such as Moloney murine leukemia virus target the promoter regions of genes, while lentiviruses, which include HIV-1, favor the bodies of active genes.[6] These specificities are largely attributable to IN-binding host factors BET (for bromodomain and extraterminal domain) proteins for Moloney murine leukemia virus[7,8] and lens epithelium–derived growth factor (LEDGF)/p75 for HIV-1.[9–11] LEDGF/p75 harbors modular protein domains that include an N-terminal PWWP chromatin-binding domain (CBD)[12,13] and a downstream IN-binding domain (IBD).[14] Constructs containing heterologous CBDs fused to the LEDGF/p75 IBD redirect HIV-1 integration to sites predictably bound by the CBD[15–17] and concordantly form the basis of the novel HIT-Seq (HIV integration targeting) genomic footprinting technique.[18] Despite their promise, the potential clinical use of such heterologous fusion constructs is complicated by the ubiquitous expression and high copy number of endogenous LEDGF/p75 protein.[11,19,20] To address this limitation, we previously reengineered the electrostatic portion of the HIV-1 IN-LEDGF/p75 binding interface to generate compatible variants of the two proteins, such that defective IN mutant proteins and viruses supported ~45% of the level of wild-type (WT) IN-LEDGF/p75 binding and ~13% of WT HIV-1 infection when presented with the reverse-charge LEDGF/p75 partner.[21] The goal of the present study was to increase the efficiency of IN mutant viral transduction in the

face of reverse-charge LEDGF/p75 mutant proteins. Using virus evolution, we selected for the novel K42E mutation in IN that increased the efficiency of the customized transduction system to ~75% of the level of WT HIV-1.

RESULTS

Experimental strategy

The details of the IN-LEDGF/p75 interaction have been elucidated using structural biology approaches.[22] IN comprises three domains: the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain.[23] The CCD harbors the primary LEDGF/p75 binding determinant, while the NTD additionally contributes to the high affinity interaction.[21,24,25] LEDGF/p75 hotspot residue Asp366 engages the loop connecting CCD α helices 4 and 5 (referred to as the α4/5 connector),[25,26] while LEDGF/p75 electropositive residues Lys401, Lys402, and Arg405 engage electronegative residues at IN NTD positions 6, 10, and 13[21] (Figure 1a).

LEDGF/p75 mutants are assessed for their ability to support HIV-1 infection using cells that are depleted of the endogenous host factor through genetic knockout[10,13] or RNA-mediated knockdown.[27,28] We previously established methods to transduce transiently transfected mouse embryo fibroblast (MEF) knockout cells with single-round HIV-Luc particles that carry the vesicular stomatitis virus G (VSV-G) glycoprotein for entry into mouse cells and the luciferase reporter gene.[10,13] Alteration of LEDGF/p75 residues Lys401, Lys402, and Arg405 to glutamates (K401E/K402E/R405E, referred to as EEE) reduced WT HIV-Luc infection by approximately fivefold, yet boosted the activity of the weakly infectious reverse-charge KK (E10K/E13K) HIV-1 IN mutant virus by approximately sixfold (from ~2% with WT LEDGF/p75 to ~13% with EEE).[21] Because the reverse-charge IBD and HIV-1 IN mutant proteins interacted in a yeast two-hybrid assay

at 45% of the efficiency of the WT pair,[21] we reasoned that transduction of LEDGF/p75 EEE-expressing MEFs by the IN KK mutant virus was limited by factors specific to the cell-based experiment. Our strategy to investigate this bottleneck to mutant viral infection was to select for the outgrowth of viruses with improved fitness over multiple rounds of replication. Several aspects of experimental design required optimization before embarking on the virus evolution experiment. Because the KK mutant virus differed from WT HIV-1 at only two nucleotide positions, it could reasonably revert back to the WT sequence over several rounds of replication. IN mutants that carried additional nucleotide changes were, therefore, tested alongside the KK mutant for transduction of WT versus EEE LEDGF/p75-expressing MEFs. As reported,[21] the transduction efficiency of the IN KK mutant virus increased approximately sixfold from expressing EEE compared with WT LEDGF/p75 (Figure 1b). Similar differential transduction levels were observed for each of the novel mutant viruses; as RR (E10R/E13R) and KR (E10K/E13R) would require six and four nucleotide changes, respectively, to revert to the WT sequence, they were earmarked for virus evolution. Mutagenesis was also used to optimize the reverse-charge LEDGF/p75 partner. To address if the electropositive Arg404 residue might interact intramolecularly with the electronegative mutant side chains in EEE (Figure 1a) and potentially interfere with mutant protein function, it was mutated to Leu in the context of EEE to yield EELE, or in the context of QEE (K401Q/K402E/R405E) to yield QELE. The KK mutant virus similarly transduced cells expressing EE (K402E/K405E), EEE, EELE, or QELE mutant LEDGF/p75 protein (Figure 1c). Because position 404 alterations did not detectably improve mutant LEDGF/p75 function, EEE was chosen for virus evolution.

Although mouse cells are efficiently transduced with appropriately pseudotyped HIV-1 particles, they do not support spreading virus replication.[29] To circumvent this limitation, TL3 cells, which were derived from CD4-positive SupT1 T cells,[27] were used for virus
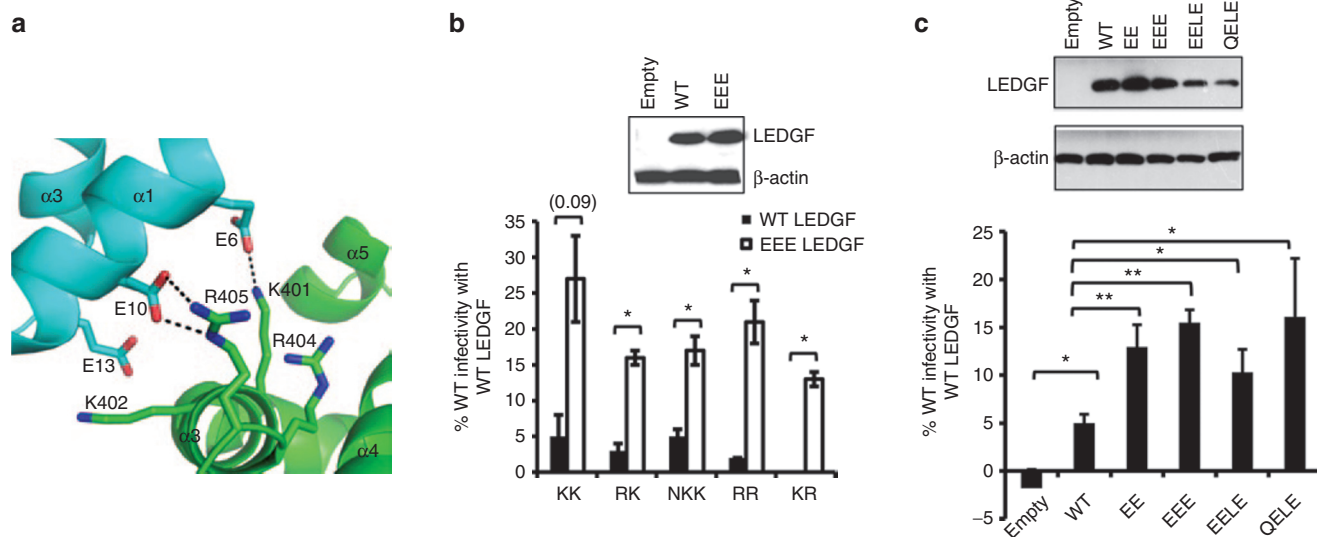

Figure 1. The IN NTD-LEDGF/p75 interaction and IN mutant viral and LEDGF/p75 protein activities. (**a**) Details of the HIV-2 IN NTD-IBD interface (chains A, B, and C from protein database code 3f9k). IN is shown in cyan, with the oxygen atoms of interacting Glu residues in red. The LEDGF/p75 IBD is in green, with the nitrogen atoms of Lys401, Lys402, Arg404, and Arg405 in blue. Salt bridge interactions between side chains are indicated by dashed line[21] (**b**) The infectivities of the indicated IN-mutant viruses were normalized to WT HIV-Luc in WT LEDGF/p75–expressing cells. The inset shows levels of WT and EEE LEDGF/p75 proteins in transfected cells following cell sorting; β-actin was monitored to control for sample loading. Two-letter codes refer to substitutions at positions 10 and 13 in HIV-1 IN. NKK, D6N/E10K/E13K. (**c**) E10K/E13K IN mutant viral infectivity in the presence of the indicated LEDGF/p75 protein was normalized to WT HIV-Luc infection of WT LEDGF/p75-expressing cells. Panel **b** and **c** results are averages of two and three independent experiments, respectively, ± SD. *$P < 0.05$ as determined by one-tail *t*-test; **$P < 0.01$. $P > 0.05$ are indicated in parentheses. EEE, K401E/K402E/R405E; HIV-1, human immunodeficiency virus type 1; IBD, IN-binding domain; IN, integrase; LEDGF, lens epithelium–derived growth factor; NTD, N-terminal domain; WT, wild type.

evolution. The level of endogenous LEDGF/p75 protein in TL3 cells is suppressed through the expression of short hairpin (sh) RNA,[27] and the cells concordantly support ~3–15% of the level of HIV-Luc transduction compared with LEDGF/p75-expressing control cells.[27,28,30] TL3 cells were transduced with puromycin-selectable retroviral vectors that expressed shRNA-resistant WT or EEE LEDGF/p75 fused to a hemagglutinin (HA) tag to monitor ectopic protein levels. Single-cell clones expressed WT and EEE HA-LEDGF/p75 proteins at levels similar to the level of endogenous LEDGF/p75 in parental SupT1 cells as assessed by blotting with anti-LEDGF/p75 and anti-HA antibodies (Figure 2a). Fluorescence-activated cell sorting was used to monitor percentages of CD4-positive cells; WT and EEE cells were 88 and 95% positive, respectively, which was similar to parental TL3 and SupT1 cells (95 and 92%, respectively). SupT1 and WT cells supported similar levels of HIV-Luc transduction, which were approximately two- to threefold greater than the levels supported by TL3 and EEE cells (Figure 2b). Although this level of infection defect for TL3 cells is somewhat milder than that reported previously, a ~27-fold defect was observed in an independent set of experiments (see below). Importantly, transduction by the IN KR mutant virus was stimulated approximately fivefold by expressing EEE compared with WT LEDGF/p75 (Figure 2c).

Selection of reverse-charge IN mutant viruses with improved replicative fitness

WT, EEE, and TL3 cells were infected with replication-competent HIV-1$_{NL4-3}$ carrying WT, KR, or RR IN at a multiplicity of infection of ~0.1,[31] and culture supernatants were monitored for evidence of virus replication over 60 days. TL3 and EEE cells supported peak WT HIV-1 replication 9 days postinfection, whereas WT LEDGF/p75 expression accelerated virus growth to yield a peak at 7 days (Figure 3a–c). HIV-1 IN mutant viral growth depended on EEE LEDGF/p75 expression; under these conditions, the peaks of KR and RR replication appeared 35 and 46 days postinfection, respectively (Figure 3c). Fresh EEE cells were infected with viruses harvested from these peaks (e1KR and e1RR) alongside WT HIV-1$_{NL4-3}$. Both e1KR and e1RR grew with significantly accelerated kinetics compared with their parental KR and RR molecular clones (compare Figure 3d with Figure 3c). Viruses e2KR and e2RR harvested from e1KR and e1RR replication peaks were passaged alongside WT HIV-1$_{NL4-3}$ onto fresh EEE and WT cells. EEE cells supported mutant viral growth delayed by only 2 days from WT HIV-1$_{NL4-3}$; by contrast,

the growth of the passaged mutant viruses was delayed from the WT virus by 17 days in WT LEDGF/p75-expressing cells (Figure 3e). Mutant viral genomes were amplified from EEE-infected cells at the peaks of e2KR and e2RR replication. The sequencing of overlapping PCR products that covered the entire genomes with the exception of the *env* gene in both cases revealed a single nucleotide A to G change at position 4353 in *pol*, which corresponded to the substitution of Glu for Lys42 (K42E) in the NTD of IN.

Two new molecular clones were made by introducing the K42E mutation into KR and RR HIV-1$_{NL4-3}$, yielding KRE and RRE, respectively. KRE and RRE grew in WT and EEE cells with kinetics that were virtually indistinguishable (delayed by at most 2 days) from serially passed e2KR and e2RR supernatants (denoted e3KR and e3RR, respectively, in Figure 4a,b). From these data, we conclude that the K42E mutation is primarily if not solely responsible for the observed increase in replication fitness of the KR and RR IN mutant viruses in EEE-expressing cells. To address if the K42E gain-of-function phenotype was observed during the early stage of HIV-1 replication that includes integration, the mutation was introduced into WT and RR single-round constructs, and knockout MEFs expressing WT, EEE, or E4 (EEE with an added K360E change)[21] LEDGF/p75 were infected with WT, RR, K42E, and RRE HIV-Luc. EEE and E4 enhanced RR HIV-Luc infectivity by approximately six- and fourfold, respectively, compared with WT LEDGF/p75 (Figure 4c). The K42E mutation interestingly reduced HIV-Luc transduction of WT LEDGF/p75-expressing cells by ~10-fold, and in the absence of other IN changes did not confer any obvious preference for EEE or E4 LEDGF/p75. Infection of EEE LEDGF/p75-expressing cells by the RR HIV-Luc mutant virus was by contrast boosted approximately threefold by the K42E mutation, such that RRE attained 75% of the level of WT HIV-Luc infectivity on cells expressing WT LEDGF/p75 (Figure 4c). We note that the K42E mutation also boosted the infectivity of the RR mutant virus in the face of WT LEDGF/p75, from about 4% without the mutation to about 24% in its presence (Figure 4c).

Biochemical analysis of recombinant protein binding and IN strand transfer activity

The results presented in Figure 4c show that the K42E mutation conferred gain-of-function to the RR mutant virus but not to WT HIV-1 under conditions that required integration and expression of the luciferase reporter gene. To test the contribution of the K42E change to WT and RR IN protein function, the K42E, RR, and RRE
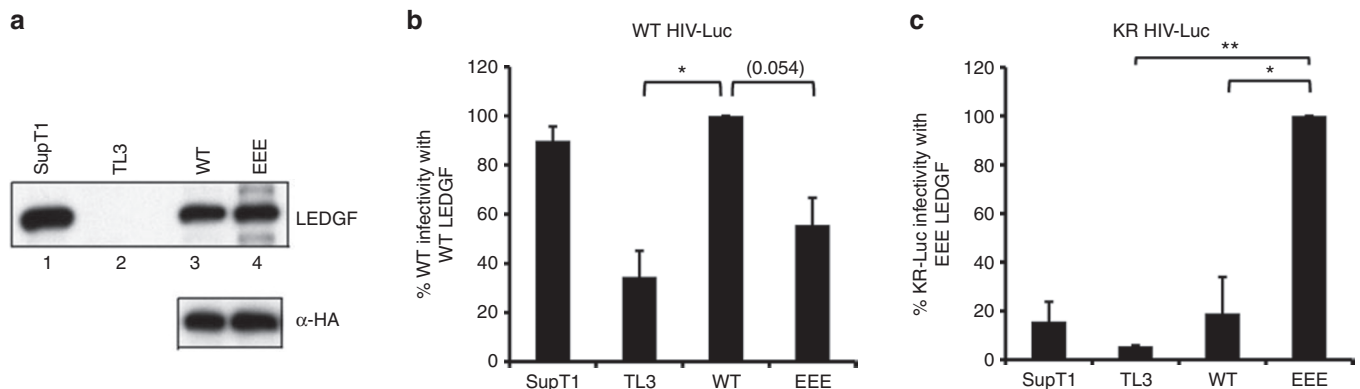
Figure 2. Construction and characterization of WT LEDGF/p75 and mutant EEE-expressing T cells. (**a**) Levels of endogenous (lanes 1 and 2) and ectopically expressed (lanes 3 and 4) LEDGF/p75 as assessed by immunoblotting. (**b**) Levels of HIV-Luc infectivity with the indicated cells, normalized to the level observed with the WT LEDGF/p75-expressing cells. (**c**) Infectivity of HIV-Luc bearing KR IN mutations with the indicated cells, normalized to the level observed with EEE-expressing cells. Panel **b** and **c** results are averages of two independent experiments ± SD. *$P < 0.05$; **$P < 0.01$. EEE, K401E/K402E/R405E; IN, integrase; KR, E10K/E13R; LEDGF, lens epithelium–derived growth factor; WT, wild type.

Figure 3. Replication kinetics of WT and IN mutant HIV-1$_{NL4-3}$ in T-cell lines. Parental (**a**) TL3 cells or (**b**) WT or (**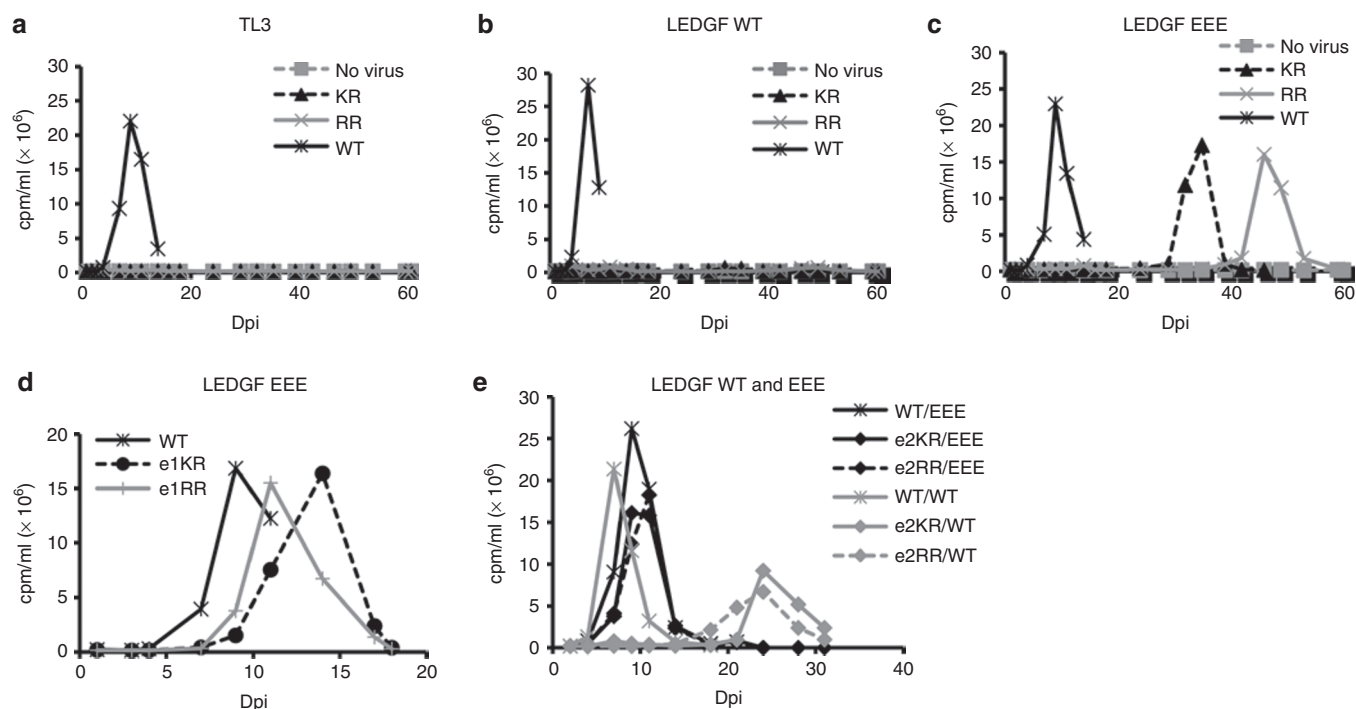c**) EEE LEDGF/p75-expressing subclones were infected with replication-competent WT, KR, or RR virus, and reverse transcriptase (RT) activity in cell supernatants was measured at the indicated time points. (**d**) Viruses harvested from EEE-expressing cells in panel **c** at the peaks of KR and RR replication (e1KR and e1RR, respectively) were passed onto fresh EEE cells, and RT values were measured every 3 or 4 days. (**e**) Viruses e2KR and e2RR collected from panel **d** replication peaks were passed onto fresh WT and EEE-expressing cells. RT values are indicated. Dpi, days postinfection; EEE, K401E/K402E/R405E; HIV-1, human immunodeficiency virus type 1; KR, E10K/E13R; LEDGF, lens epithelium–derived growth factor; RR, E10R/E13R; WT, wild type.
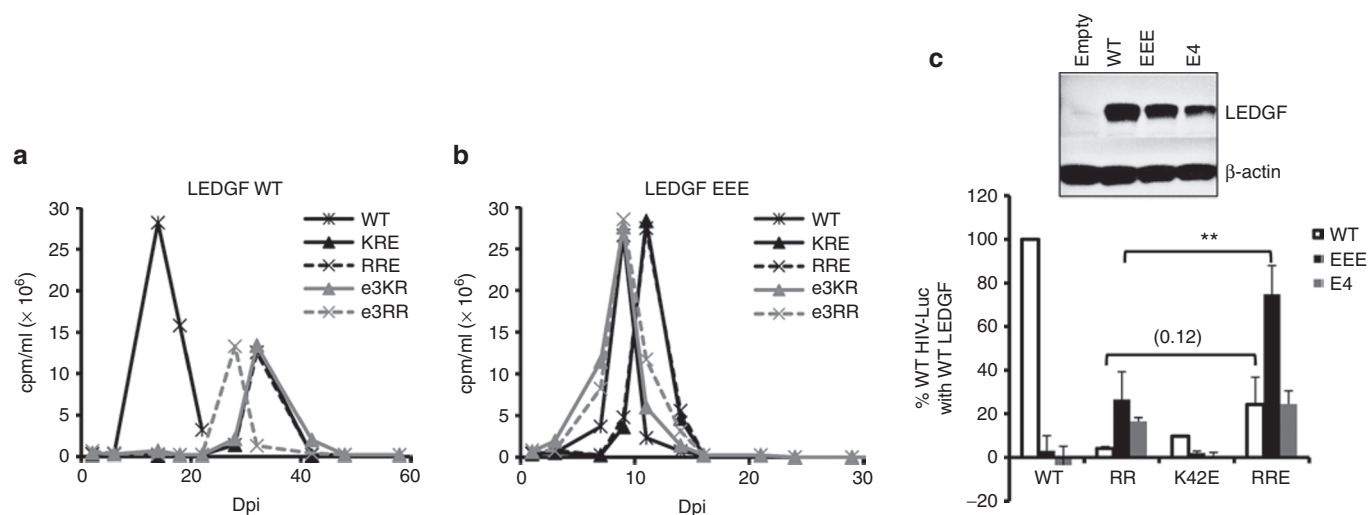

Figure 4. The K42E mutation accounts for the improved replication fitness of KR and RR mutant viruses. (**a**) TL3 cells expressing WT LEDGF/p75 were infected with WT, KRE, or RRE HIV-1$_{NL4-3}$ alongside e3KR and e3RR supernatants harvested from replication peaks in panel **e** from Figure 3. Reverse transcriptase activity was determined at the indicated time points. (**b**) Same as in panel **a**, except that EEE cells were infected. (**c**) WT, K42E, RR, and RRE HIV-Luc transduction of knockout MEFs transfected with the indicated empty or WT, EEE, or E4 LEDGF/p75 expression vector. Infectivity of WT HIV-Luc in the presence of WT LEDGF/p75 was set to 100%; other values are mean ± SD for *n* = 2 independent experiments. Statistical comparisons of RR versus RRE viruses on cells expressing WT and EEE LEDGF/p75 are indicated. **P < 0.01. EEE, K401E/K402E/R405E; HIV-1, human immunodeficiency virus type 1; KR, E10K/E13R; LEDGF, lens epithelium–derived growth factor; MEF, mouse embryo fibroblast; RR, E10R/E13R; WT, wild type.

mutations were engineered into a hexahistidine (His$_6$)-tagged IN expression construct, and proteins purified following their expression in bacteria were tested in *in vitro* LEDGF/p75 binding and IN activity assays. Binding of IN-His$_6$ to WT and EEE LEDGF/p75 was determined using a Ni^{2+}-nitrilotriacetic acid pull-down assay.[21] The K42E mutation reduced WT LEDGF/p75 binding by approximately

twofold, whereas neither RR nor RRE IN bound a detectable level of WT LEDGF/p75 (Figure 5a, lanes 1–11; quantified in Figure 5b). As reported,[21] WT IN did not detectably bind EEE LEDGF/p75 protein. Compared with the level of WT LEDGF/p75 protein bound by WT IN, K42E, RR, and RRE IN bound EEE LEDGF/p75 protein at ~42, 11, and 15% efficiency, respectively (Figure 5a, lanes 12–16, and Figure 5b).

IN strand transfer activity was assayed using an oligonucleotide substrate that modeled the U5 end of HIV-1 DNA and a circular plasmid DNA target. Mimicking its function during virus infection, recombinant IN can integrate a pair of oligonucleotide substrates into opposing strands of target DNA with a 5-bp spacing, which after deproteination yields a linear concerted DNA recombination product.[21] For reasons that are not entirely clear, HIV-1 IN yields substantial half-site integration products *in vitro*, which result from a failure to either engage or integrate a second molecule of substrate oligonucleotide. In agarose gels, such half-site products comigrate with the open circular form of the target DNA plasmid isolated from *Escherichia coli*. In the absence of LEDGF/p75, IN displayed a basal level of half-site strand transfer activity (Figure 5c, lanes 1–5). The addition of LEDGF/p75 significantly stimulated IN half-site and concerted strand transfer activities (compare lane 6 with lane 2 in Figure 5c).[21] Because the concerted integration product becomes retargeted by half-site integration events, we note its migration varies depending on the overall level of strand transfer activity. K42E, RR, and RRE INs supported about 83, 53, and 18% of the level of WT IN concerted integration activity in the presence of WT LEDGF/p75 (Figure 5c, lanes 6–9; quantified in Figure 5d). EEE LEDGF/p75 stimulated about 17% of WT IN concerted integration activity,

though it failed to appreciably stimulate the K42E IN mutant protein (Figure 5c, lanes 10 and 11). The mutant integration cofactor stimulated RR and RRE concerted integration activities to ~55 and 78% of the levels observed for WT IN with WT LEDGF/p75 (Figure 5c,d).

Stimulation of IN mutant viral DNA synthesis and integration by the K42E mutation

Although our biochemical analysis revealed a gain-of-function for RR IN from the K42E mutation in the presence of LEDGF/p75 EEE protein, the magnitude of the differential strand transfer activity effect appeared less than that observed during virus infection (compare Figures 5d and 4c). The K42E change moreover only marginally reduced WT LEDGF/p75-dependent IN strand transfer activity (Figure 5d), yet reduced HIV-Luc infectivity by ~10-fold (Figure 4c). To further investigate the molecular bases for the viral infection phenotypes, reverse transcription and integration were analyzed by quantitative PCR using single-round WT, RR, K42E, and RRE HIV-Luc viruses and TL3, WT, and EEE T cells. PCR primers and probes were designed to amplify total HIV-1 DNA after the second template switch of reverse transcription (so-called late reverse transcription or LRT products) and nuclear DNA subsets that included two-long terminal repeat (2-LTR) containing circles and integrated proviruses.
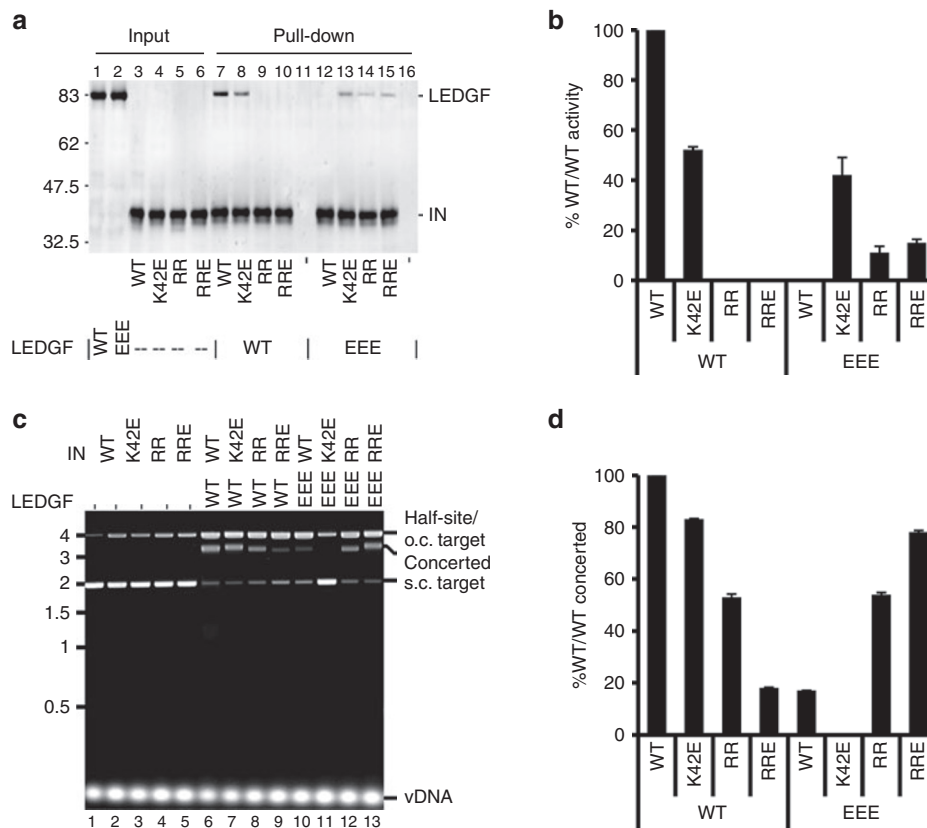

Figure 5. Effects of mutations on protein binding and IN strand transfer activity. (**a**) IN-LEDGF/p75 binding as assessed by Ni-nitrilotriacetic acid pull-down. Lanes 1–6 show 100% of the levels of input proteins. Pull-down reactions with WT LEDGF/p75 were loaded in lanes 7–11, whereas parallel reactions with EEE LEDGF/p75 were analyzed in lanes 12–16. IN was omitted from the reactions loaded in lanes 11 and 16. The gel was stained with Coomassie blue. (**b**) Quantitation of WT and EEE LEDGF/p75 recovery by the indicated IN protein for two experiments, expressed as averages ± SEM. The results were normalized to the level of WT LEDGF/p75 recovery by WT IN (set to 100%). (**c**) IN strand transfer activities. The migration positions of the oligonucleotide viral DNA substrate, supercoiled (s.c.) and open circular (o.c.) forms of pGEM-3 target DNA plasmid, as well as half-site and concerted integration reaction products, are shown alongside the agarose gel image. IN was omitted from the reaction loaded in lane 1. (**d**) Quantitation of concerted integration activities of IN mutant proteins in the presence of WT or EEE LEDGF/p75. The results (averages ± SEM for *n* = 2 experiments) were normalized to the level of WT IN activity in the presence of WT LEDGF/p75, which was set to 100%. EEE, K401E/K402E/R405E; IN, integrase; LEDGF, lens epithelium–derived growth factor; WT, wild type.

DNA was isolated from cells at 8, 24, and 48 hour postinfection, corresponding to the peaks of WT HIV-1 LRT, 2-LTR circle, and provirus formation, respectively.[32,33]

WT LEDGF/p75 expression in this series of experiments boosted HIV-Luc infection of TL3 cells ~27-fold, while EEE expression enhanced RR and RRE transduction ~14- and 9-fold over the levels supported by WT LEDGF/p75 (Figure 6a). As expected,[10,27] WT HIV-1 DNA synthesis was independent of the level of LEDGF/p75 expression (Figure 6b). By contrast, the RR and K42E IN mutations caused approximately three- to fivefold reverse transcription defects. Because mutations in IN can affect HIV-1 replication at multiple steps, we previously established a classification system to delineate those mutations that specifically affect integration (class I) from those that additionally affect other steps in the viral life cycle (class II).[34] The most common pleiotropic defect among class II HIV-1 IN mutant viruses is reverse transcription, and K42E and RR were accordingly typed as class II IN mutant viruses. Combining the K42E and RR mutations interestingly restored IN mutant reverse transcription to ~70% of the level of WT HIV-Luc (Figure 6b).

Perturbation of HIV-1 integration through class I IN mutations,[35,36] IN strand transfer inhibitors,[37] or depletion of integration cofactors[10,27] yields transient increases in the levels of nuclear side-product DNAs such as 2-LTR circles, and WT HIV-Luc 2-LTR circles were accordingly increased by ~2.4-fold in TL3 cells from the level observed in WT LEDGF/p75-expressing cells (Figure 6c). Because the levels of IN mutant RRE 2-LTR circles were similar to the level for WT HIV-Luc in WT LEDGF/p75-expressing cells, the mutant viral

DNA efficiently accessed cell nuclei. The integration of RRE viral DNA, however, strictly depended on the presence of the reverse-charge EEE LEDGF/p75 cofactor (Figure 6d).

DISCUSSION

Although retroviruses are among the most popular vehicles for introducing transgenes, adverse genotoxic effects from insertional mutagenesis are a significant cause for concern in human gene therapy (reviewed in ref. [38]). Vectors derived from lentiviruses such as HIV-1 appear less genotoxic than γ-retroviral predecessors, which likely reflects the differential targeting of host cell chromatin by these two types of retroviruses.[6,38] Despite this benefit, the observed growth advantage of particular cell clones reveals that lentiviral vectors, though likely safer than γ-retroviral–based counterparts, may not be free of insertion-based side effects.[39] Various approaches are being used to increase the safety of lentiviral gene therapy vectors, including the modification of *cis*-acting elements to reduce the potential for read-through transcription of growth-promoting cellular genes.[40] Alteration of the sites of DNA integration is another strategy to increase the safety of gene therapy vectors. Modification of HIV-1 IN with site-specific DNA binding elements can provide up to an ~10-fold increase in integration site preference, but with a concomitant ~10-fold decrease in vector titer.[5] Global integration retargeting through heterologous CBDs fused to the lentivirus-specific targeting host factor LEDGF/p75 IBD can by contrast proceed without significant loss in virus titer[15–17] and reveal novel, genome-wide regions of CBD binding.[18] Attempts to
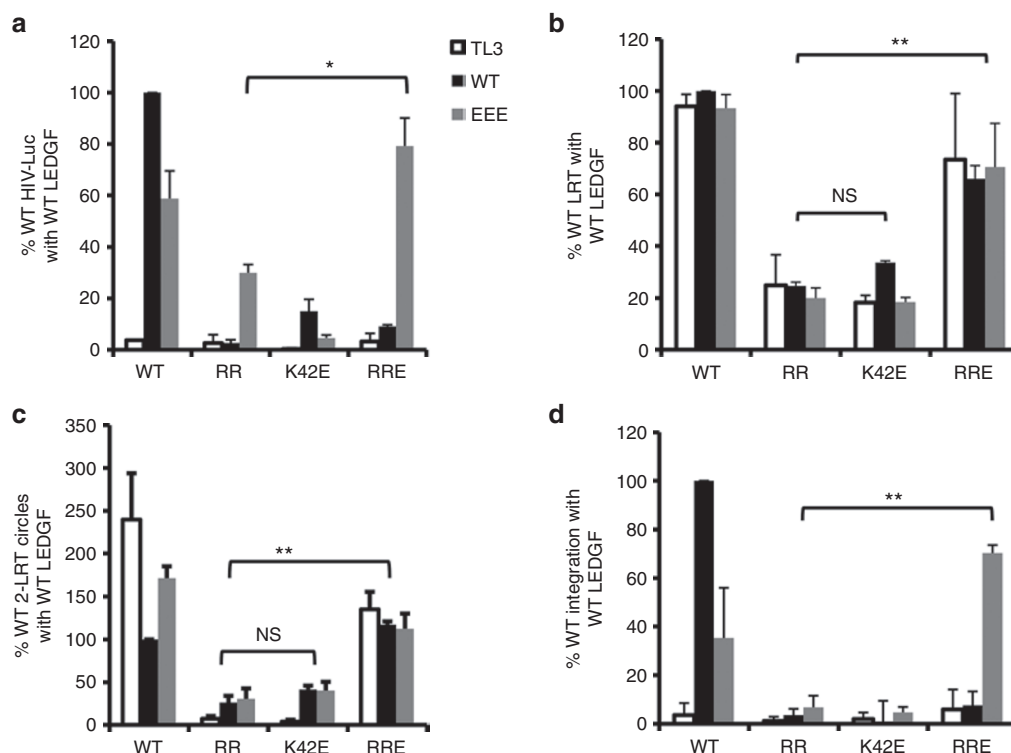

Figure 6. WT and IN mutant viral reverse transcription and integration activities. (**a**) Levels of WT, RR, K42E, and RRE HIV-Luc infectivity using the indicated T-cell line. Results were normalized to the level of WT HIV-Luc transduction in the presence of WT LEDGF/p75, which was set to 100%. (**b**) Levels of LRT product formation from the infections in panel **a**, normalized to the level of WT HIV-Luc DNA synthesis in cells expressing WT LEDGF/p75 (set at 100%). (**c**) Levels of 2-LTR circle formation from panel **a** infections, normalized to the WT virus in cells expressing WT LEDGF/p75. (**d**) Virus integration levels were normalized to the level of WT HIV-Luc virus in cells expressing WT LEDGF/p75, which was set at 100%. Values in panels **a**–**d** represent averages ± SD for two independent experiments. Panel **a** and **d** statistical comparisons evaluate RR versus RRE virus in EEE LEDGF/p75-expressing cells, whereas those in panels **c** and **d** compare the activities of the indicated viruses across cell lines. *$P < 0.05$; **$P < 0.01$. EEE, K401E/K402E/R405E; IN, integrase; LEDGF, lens epithelium–derived growth factor; NS, not significant; WT, wild type.

utilize heterologous LEDGF/p75 fusion proteins in the clinical setting must circumvent the relatively high copy number of endogenous LEDGF/p75 protein in target cells.[20] We previously developed defective IN mutant viruses whose transduction efficiencies were restored to ~13% of maximum in the face of a complementary, reverse-engineered LEDGF/p75 mutant protein.[21] In this report, we have increased the efficiency of this system to ~75% of the level of unmodified virus in cells that express WT LEDGF/p75 protein.

The importance of the K42E gain-of-function mutation is evident by its independent selection through the serial passage of defective IN mutant viruses RR and KR in LEDGF/p75 EEE-expressing cells (Figures 3 and 4). Strikingly, the K42E mutation by itself reduced HIV-1 titer ~7- to 10-fold (Figures 4c and 6a), which is primarily attributable to a reverse transcription defect (Figure 6b). The inability for EEE LEDGF/p75 to support K42E IN strand transfer activity *in vitro* (Figure 5) likely accounts for the added K42E IN mutant viral defect in EEE-expressing cells. Although we previously described the selection of a second-site HIV-1 IN mutation (T125A) that restored function to replication-defective P109S mutant virus, the T125A change on its own conferred no obvious growth disadvantage.[41] The K42E mutation to the best of our knowledge is the first example of an HIV-1 IN gain-of-function mutation that by itself severely limits virus activity, though we do note the description of pleiotropic gain-of-function mutations in other biological systems.[42]

The K42E mutation impressively boosted the level of RR IN mutant reverse transcription, from ~20 to ~70% of the WT, even though the change on its own conferred an approximately fivefold reverse transcription defect (Figure 6b). As the reverse transcription boost is LEDGF/p75 independent, we envisage it in large part drives the gain in IN mutant function in the face of WT LEDGF/p75; it is noteworthy that this effectively caps the specificity of the RRE virus for EEE over WT LEDGF/p75 at approximately three- (Figure 4c) to ninefold (Figure 6a). This degree of selectivity is nevertheless predicted to significantly improve the efficiency of LEDGF-based retargeting strategies in cells that express the endogenous integration cofactor.[15,17,43] Because the RRE mutant virus integrated in cells that express LEDGF/p75 EEE at ~70% of the efficiency of WT HIV-1 in WT LEDGF/p75-expressing cells (Figure 6d), we conclude that the RRE virus integrates with the WT efficiency under these conditions. The ~30% impairment in IN mutant RRE reverse transcription would seem to uniquely limit the transduction efficiency of the optimized IN-LEDGF/p75 reverse-charge system (Figure 6).

HIV-1 IN and reverse transcriptase (RT) proteins can interact *in vitro*, with the IN C-terminal domain harboring key RT-interacting residues.[44] It is unclear how changes in the IN NTD might disrupt and then functionally restore reverse transcription, though long-range effects of the mutations on RT-IN binding seems one possibility. The molecular basis for the integration gain-of-function phenotype was investigated by analyzing the position of the Lys42 side chain in HIV-1 IN crystal structures. Because the two-domain NTD-CCD construct from the IBD cocrystal structure was based on HIV-2 IN,[21] a molecular model was constructed by aligning the common CCD element from the HIV-1 IN CCD-LEDGF/p75 structure[25] with an HIV-1 IN NTD-CCD two-domain structure.[45] As anticipated[21] (Figure 1a), HIV-1 IN residues Asp6, Glu10, and Glu13 approach LEDGF/p75 residues Lys401, Lys402, and Arg405 in the overlay. Lys42 interacts intramolecularly with CCD residue Asp167, which forms part of the critical α4/5 connector (IN residues 166-171) engaged by LEDGF/p75 residue Asp366 (ref. 25) (Figure 7). As numerous changes in α4/5 connector residues affect the HIV-1 IN-LEDGF/p75 interaction,[26,46,47] we speculate that altered connector loop position could determine the

influence of the K42E mutation on the virus–host interaction. We also note that IBD engagement shifts the position of the Asp167 carboxylate toward LEDGF/p75 residue Lys360 (Figure 7). It, therefore, seems possible that the K42E mutation could influence the protein interaction through altering the Asp167-Lys360 contact. Because our data emphasizes the benefit of the K42E mutation in the backdrop of reverse-charge substitutions at multiple other LEDGF/p75 and IN positions, additional structural work with K42E IN mutant proteins will be required to fully appreciate the molecular basis of the integration gain-of-function phenotype. We note that structural analysis of HIV-2 IN might be of limited benefit, as the analogous position 42 amino acid, glutamine, is not positioned to interact with Glu167 in the NTD-CCD–IBD cocrystal structure (not shown).

Although site-specific integration has yet to be reported using LEDGF/p75 fusion constructs, HIV-1 vectors directed to integrate into heterochromatin via HP1α[15,16] or CBX1[17,43] CBDs express remarkably well considering overall random targeting of genes and promoter regions. Heterochromatin-directed retargeting is, therefore, one potential avenue toward safety-improved lentiviral vectors. Retargeting, by definition, requires the presence of the LEDGF/p75 fusion protein in target cells, which has been accomplished *ex vivo* through transient or stable expression.[15–18,43] Results of preliminary experiments suggest that piggy backing LEDGF/p75 into the virion through its fusion with the viral Vpr accessory protein fails to effectively complement the infection defect of mouse knockout MEFs, so this strategy seems unlikely to work. Potential translational applications are accordingly currently limited to *ex vivo* settings, and
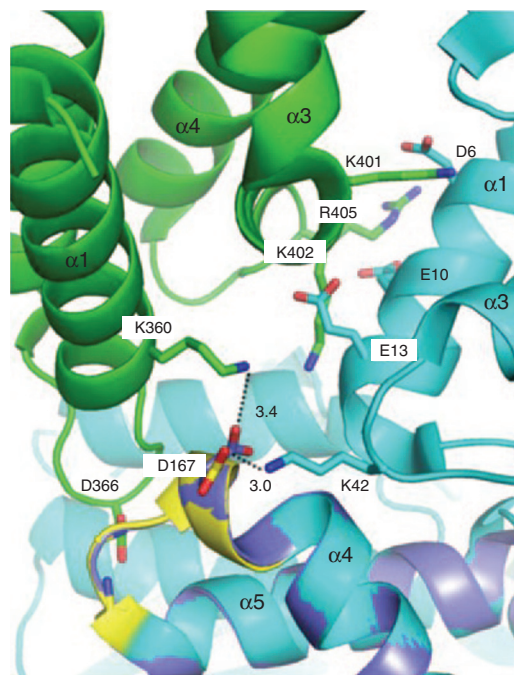

Figure 7. Potential impact of the K42E mutation on reverse-charge HIV-1 IN and LEDGF/p75 function. The CCD (blue) from molecule A of the HIV-1 IN CCD-LEDGF/p75 IBD cocrystal structure (protein database code 2bj4)[25] was aligned with the CCD (cyan) of molecule B from the HIV-1 NTD-CCD 2-domain structure (pdb code 1k6y)[45] using PyMOL. Distances of ≤4.0 Å between Lys42, Asp167, and Lys360 side chains in the two structures are indicated by dashed lines. The CCD α4/5 connector (IN residues 166–171) is highlighted in yellow; other coloring is the same as in Figure 1. CCD, catalytic core domain; HIV-1, human immunodeficiency virus type 1; IBD, IN-binding domain; IN, integrase; LEDGF, lens epithelium–derived growth factor; NTD, N-terminal domain.

heterochromatin-based retargeting through transient expression of CBX1-LEDGF from electroporated RNA has been shown to restore NADPH-oxidase activity in a cell line model.[43] The incorporation of components from our optimized reverse-charge HIV-1 IN-LEDGF/p75 system would be expected to increase the specificity of not only targeted integration under similar translational settings, but also high-resolution HIT-Seq maps in cells that express endogenous LEDGF/p75 protein.

MATERIALS AND METHODS

DNA constructs

Plasmids pNL43/XmaI, pUC19.2LTR, pNLX.Luc.R–,[31] pCG-VSV-G, pCG-gagpol, pIRES2-eGFP, pIRES2-eGFP-LEDGF-HA,[10] pKBIN6Hthr,[48] and pFT-1-LEDGF[19] were previously described. Plasmid pLPCX-HA-LEDGF/p75 was built by inserting PCR-amplified HA-LEDGF/p75 into pLPCX vector DNA (Clontech Laboratories, Mountain View, CA). Mutations were introduced by PCR using *Pfu* Ultra DNA polymerase (Agilent Technologies, Santa Clara, CA), and plasmids were sequenced to verify the presence of mutations and absence of unwanted secondary changes.

Cells, viruses, and infection assays

HEK293T and MEF cells were grown in Dulbecco's modified Eagle medium supplemented to contain 10% fetal bovine serum and 100 IU/ml penicillin–streptomycin. SupT1 and TL3 cells were maintained in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum and 100 IU/ml penicillin–streptomycin. Reexpression of LEDGF/p75 in TL3 cells was accomplished by transduction with pLPCX-HA-LEDGF/p75 expression vectors that carried shRNA-resistant complementary DNAs with seven synonymous changes within the 21-nt shRNA target region. Vectors were produced in HEK293T cells by transfecting 10 μg pLPCX-HA-LEDGF/p75, 3 μg pCG-gagpol, and 3 μg pCG-VSV-G using Fugene (Promega Corporation, Madison, WI). Supernatants collected 24 and 48 hours after transfection were concentrated 20-fold by ultracentrifugation at $150,000\,g$ for 3 hours at 4 °C. TL3 cells (1×10^5) were transduced with concentrated supernatants, followed by the addition of puromycin (0.15 μg/ml) 48 hours later. Cells were seeded for single-cell cloning 48 hours thereafter. Western blotting was performed as previously described.[10,13]

HIV-1$_{NL4-3}$ viruses were generated by cotransfecting HEK293T cells with pNLX.Luc.R– and pCG-VSV-G (single-round HIV-Luc) or pNL43/XmaI (replication competent) as previously described;[31] levels of virus release into the cell supernatant were determined by exogenous RT assay.[31] SupT1 (1×10^5 per well of a 24-well plate) were infected in duplicate with equal RT-cpm (2.5×10^6) of WT or IN mutant HIV-Luc for 2 hours. Cells harvested 48 hours from the start of infection were processed for luciferase activity assays in duplicate, and results were processed as average relative light units per total microgram protein in cell extracts as determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA).[31] SupT1 cells (10^6) were infected with equal RT-cpm (10^6) of WT or IN mutant HIV-1$_{NL4-3}$. MEF cells (5×10^6) were transfected with 10 μg of pIRES2-eGFP expression vector using MEF Nucleofector solution 1 (Lonza, Allendale, NJ) according to the manufacturer's instructions. Transfected cells were plated in 10-cm dishes for 24 hours prior to fluorescence-activated cell sorting to select for green fluorescent protein–positive transfectants. After sorting, cells (2×10^4) were seeded in wells of a 24-well plate prior to infection with equal RT-cpm (10^6) of WT or IN mutant HIV-Luc in duplicate. Relative light units per total microgram protein values from cells transfected with empty vector pIRES2-eGFP were subtracted from experimental samples.[10,13]

Recombinant proteins and in vitro biochemistry

His$_6$-tagged HIV-1 IN proteins were expressed from pKBIN6Hthr in *E. coli* and purified as previously described.[49] LEDGF/p75 proteins expressed from pFT-1-LEDGF in bacteria were purified as described.[19] His$_6$ tag was removed from IN by cleavage with thrombin before activity assays, and conditions for the Ni-nitrilotriacetic acid pull-down and IN strand transfer assays were as described.[49]

Quantification of HIV-1 reverse transcription and integration

SupT1 cells were washed following 2 hours of infection with DNase-treated HIV-Luc, and DNA was isolated from cells using the DNeasy blood and tissue kit (Qiagen, Germantown, MD). HIV-1 LRT products were amplified in duplicate reactions containing 20–30 ng of DNA, 0.2 μmol/l primers AE2963/

AE4422, 0.1 μmol/l probe AE2965, and 1 × QuantiTect Probe PCR Master Mix. Reactions were incubated at 50 °C for 2 minutes, 95 °C for 15 minutes, and then cycled 40 times at 94 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. Serial dilutions of pNLX.luc.R– in uninfected cell DNA generated the LRT standard curve. The same amplification conditions were used for 2-LTR circles with primers AE4450/AE4451 and probe AE4452; serial dilution of plasmid pUC19.2LTR in uninfected cell DNA generated the 2-LTR circle standard curve.[10,32,50] Integration efficiency was quantitated by Alu-PCR as previously described.[50] First-round PCRs amplified sequences between integrated virus and chromosomal Alu elements using primers AE3014/AE1066 at 94 °C for 5 minutes, followed by 18 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C, and 5 minutes at 70 °C; reactions were terminated following a final 10-minute extension at 72 °C. Second-round DNA (1–2 μl) amplification utilized primers AE3013/AE990 and probe AE995 under the aforementioned quantitative PCR cycling conditions. Values obtained from parallel first-round reactions that omitted Alu-specific AE1066 primer were subtracted from second-round quantitative PCR values. The Alu-PCR standard curve was made by step-wise dilution of DNA from WT HIV-Luc-infected cells in uninfected cell DNA. To account for potential plasmid DNA carryover from transfected cell supernatants, parallel infections were conducted in the presence of RT inhibitors (100 μmol/l azidothymidine or 10 μmol/l efavirenz), and resulting quantitative PCR values were subtracted from experimental LRT, 2-LTR circle, and Alu integration samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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