Phage Display-directed Discovery of LEDGF/p75 Binding Cyclic Peptide Inhibitors of HIV Replication

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The interaction between the human immunodeficiency virus (HIV) integrase (IN) and its cellular cofactor lens epithelium-derived growth factor (LEDGF/p75) is crucial for HIV replication. While recently discovered LEDGINs inhibit HIV-1 replication by occupying the LEDGF/p75 pocket in IN, it remained to be demonstrated whether LEDGF/p75 by itself can be targeted. By phage display we identified cyclic peptides (CPs) as the first LEDGF/p75 ligands that inhibit the LEDGF/p75–IN interaction. The CPs inhibit HIV replication in different cell lines without overt toxicity. In accord with the role of LEDGF/p75 in HIV integration and its inhibition by LEDGINs, CP64, and CP65 block HIV replication primarily by inhibiting the integration step. The CPs retained activity against HIV strains resistant to raltegravir or LEDGINs. Saturation transfer difference (STD) NMR showed residues in CP64 that strongly interact with LEDGF/p75 but not with HIV IN. Mutational analysis identified tryptophan as an important residue responsible for the activity of the peptides. Serial passaging of virus in the presence of CPs did not yield resistant strains. Our work provides proof-of-concept for direct targeting of LEDGF/p75 as novel therapeutic strategy and the CPs thereby serve as scaffold for future development of new HIV therapeutics.

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INTRODUCTION

The majority of currently available antiretrovirals target the enzymatic activity of the virus-encoded enzymes: reverse transcriptase (RT), integrase (IN), and protease. Since human immunodeficiency virus (HIV) has a short life cycle with a highly error prone RT, the targeted proteins can rapidly evolve toward drug resistance which jeopardizes the long-term efficacy of the drugs. Identification of novel therapeutic targets thus remains a major priority in antiviral research. Because of its limited genome, HIV is dependent on host cell machineries and proteins to complete its replication cycle. In theory, small molecules or peptides that bind to the selected host cofactor may impede virus replication. For instance maraviroc, a CCR5 antagonist was recently approved for patient treatment.[1] Maraviroc binds to the CCR5 coreceptor and blocks HIV-1 entry.[2]

Lens epithelium-derived growth factor (LEDGF)/p75 seems well suited for such efforts because it has a distinct interaction interface with HIV IN that can accommodate small molecule inhibitors.[3,4] LEDGF/p75 is a chromatin-associated protein that tethers the preintegration complex to the host chromatin through its direct interaction with IN thereby supporting HIV replication.[5] Additionally, LEDGF/p75 has been associated with cancer and autoimmunity and therefore LEDGF/p75 seems to play a crucial role at the center of multiple pathologic processes.[6,7]

A proof-of-concept approach using overexpression of the $LEDGF_{325–530}$ fragment containing the IN-binding domain (IBD), demonstrated that the LEDGF/p75–IN interaction is a potential target for the development of small molecule inhibitors of protein–protein interaction (PPI).[8,9] The discovery of LEDGINs,[10] supports the new paradigm in antiviral research based on targeting intracellular virus–host interactions instead of viral enzyme activities. Moreover, recently we have shown that overexpression of the $LEDGF_{325–530}$ fragment of LEDGF/p75 protects primary $CD4^+$ T-cells from HIV mediated cell killing and inhibits HIV propagation *in vivo*.[11] Therefore, these evidences support the idea that a reciprocal approach to identify small molecules or peptides that bind to LEDGF/p75 might indeed be a valuable alternative. Targeting the cellular partner of the virus–host interaction might come with an additional benefit of an increased genetic barrier to resistance development.

However, whether molecules can be designed or identified to bind to LEDGF/p75 instead of IN and inhibit HIV replication

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remained to be answered. We designed a phage display strategy to identify peptide ligands that bind specifically to the C-terminal fragment of LEDGF/p75 (amino acid 325–530; $LEDGF_{325–530}$) containing the IBD. Random peptide phage display is a widely used technology to identify peptides inhibiting PPI in the context of drug discovery[12] or vaccine development.[13,14] Here, we report the identification of small peptide inhibitors (CP64 and CP65) of the LEDGF/p75–IN interaction and HIV replication that block provirus formation. Tryptophan is a critical residue for the activity of the peptides. Saturation transfer difference (STD) NMR confirms that the peptides bind specifically to LEDGF/p75 and not to IN. We failed to select virus resistant to inhibition by the CPs, ligands of a cellular protein. Our results validate LEDGF/p75 as a genuine antiviral target.

RESULTS

Phage display reveals peptides that inhibit the interaction between LEDGF/p75 and HIV IN

Today, we can effectively inhibit HIV replication by disrupting the LEDGF/p75–IN interaction by LEDGINs.[10] This evidence strongly supports the LEDGF/p75–HIV IN interaction interface as a genuine therapeutic target. LEDGINs bind to the LEDGF/p75 binding pocket in IN. To increase our knowledge about the versatility of this antiviral target and investigate whether it can be effectively manipulated by ligands that bind to the cellular protein LEDGF/p75 without side-effects, we carefully designed a phage display strategy to identify small peptides binding to the C-terminal fragment of LEDGF/p75, $LEDGF_{325–530}$ (**Figure 1**). The C-terminal fragment of LEDGF/p75 was used because it contains the IBD.[3] To isolate peptides that specifically bind to $LEDGF_{325–530}$, we first depleted the M13-derived random peptide phage libraries (linear 7-mer and 12-mer, cyclic 7-mer) by preabsorbing the libraries on beads coated with wild-type HIV-1 IN. Phages remaining in the supernatant were used for positive selection rounds with $LEDGF_{325–530}$-coated beads (**Figure 1**). After three rounds of positive selection, phages were titered and individually tested for specific binding to $LEDGF_{325–530}$.

Using phage enzyme-linked immunosorbent assay (ELISA), we selected 50 clones that specifically interact with $LEDGF_{325–530}$ (**Supplementary Table S1**). All 50 clones were sequenced for the peptide-coding regions and found to represent 10 different peptides (**Table 1**). In the linear 7-mer library, we isolated two different peptides; interestingly one was selected multiple times. From the linear 12-mer library, only one peptide sequence was isolated, but 30 times. From the cyclic 7-mer library, 13 clones representing 7 different CPs were identified showing substantial sequence similarity (**Table 1**). Several of the isolated CPs have a common motif (V^M/xGHP^L/$_X$W) without linear or conformational homology to IN when examined using the 3DEX software program.[15]

In contrast to linear peptides, cyclic peptides (CPs) have the following favorable characteristics:[16] (i) they are inherently stable and are less prone to proteolytic degradation by exopeptidases; (ii) their reduced flexibility is an advantage for the interaction with the potential molecular target; (iii) reduced flexibility facilitates structural studies. Moreover, most of the CPs described in this study have a common motif indicating that the different CPs bind at the same spot on LEDGF/p75. Therefore, we selected the sequences of three CPs namely CP63, CP64, and CP65 (**Table 1**, sequences highlighted in gray) for further analysis.

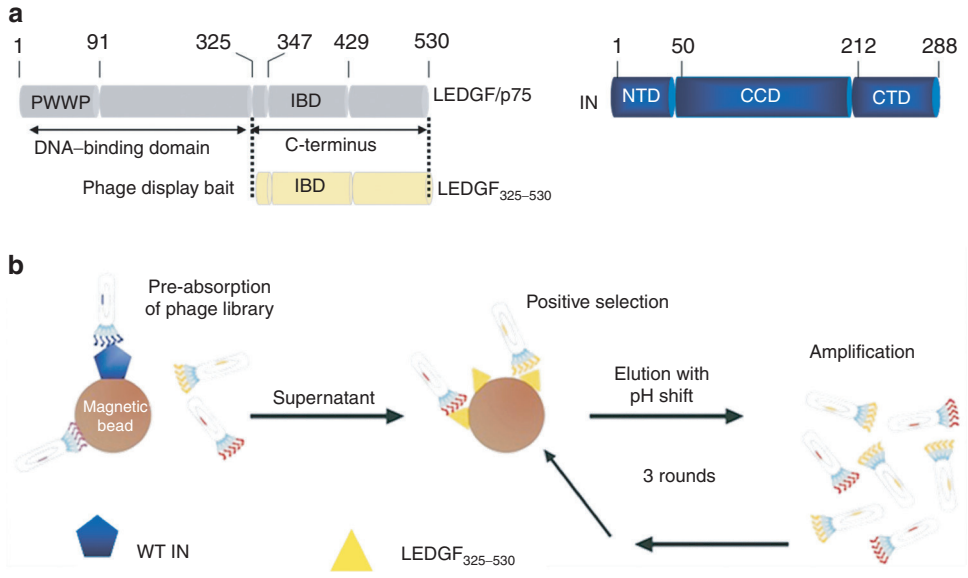

Figure 1 Phage display biopanning strategy to identify lens epithelium-derived growth factor (LEDGF)/p75 ligands. (**a**) Schematic representation of domains organization of LEDGF/p75 (gray) and integrase proteins (blue). The PWWP domain at the N-terminus of LEDGF/p75 provides a critical chromatin recognition function. The C-terminus (in yellow), represented as $LEDGF_{325–530}$, contains the integrase-binding domain (IBD). HIV-1 integrase (IN) (blue) is composed of three domains: the N-terminal domain (NTD), the catalytic core domain (CCD) and the C-terminal domain (CTD). (**b**) Phage display peptide libraries were preabsorbed using paramagnetic beads coated with wild-type HIV-1 integrase (WT IN) to deplete binders to beads and to WT IN (purple, blue phages; preabsorption). Supernatant with residual phages was subsequently used to isolate specific ligands of $LEDGF_{325–530}$ immobilized on beads (red, yellow phages; positive selection). Bound phages were eluted via pH shift, amplified and used for another round of selection. Overall three rounds of selections were performed. HIV, human immunodeficiency virus.

The selected CPs inhibit the LEDGF/p75–IN interaction *in vitro*

The three selected active CPs (CP63-65) were synthesized to high purity (>90%) and their capacity to inhibit the interaction between IN and LEDGF/p75 was tested in an AlphaScreen assay.[10] All the CPs were tested in parallel and inhibited the interaction between IN and LEDGF/p75 with IC_{50} values in the mid-micromolar range (20–60 µmol/l) (**Table 2**). Only the most active peptide, CP63 (IC_{50} 19.37±7.69 µmol/l), inhibited the interaction of LEDGF/p75 with its cellular binding partner JPO2,[17] which could potentially lead to cellular toxicity.

Next, we tested CP64 and CP65 with their respective mutant peptides in an *in vitro* IN enzymatic activity assay to investigate whether they interfere with the catalytic activity of HIV-1 IN. The tested peptides did not inhibit the catalytic activity of HIV-1 IN consistent with their presumptive binding to LEDGF/p75 (**Supplementary Table S2**). These data corroborate the phage biopanning ELISA results in which most of the peptides strongly interact with LEDGF/p75 but not with IN (**Supplementary Table S1**).

In search of a mutant control, the single tryptophan residue of the CPs was selected and substituted to alanine since initial STD NMR analysis (data not shown) suggested that this residue has multiple interactions with residues of LEDGF/p75. Since both CP63 and CP64 have similar sequences except one amino acid, we only performed the mutant analysis with CP64 and CP65. Therefore, both C64m and CP65m were synthesized in parallel with the respective active peptides and with comparable purity (>90%) and their inhibitory activity was evaluated using an AlphaScreen assay. Both CP64m and CP65m that represent CP64 and CP65 without the tryptophan residue lost activity, indicating that the tryptophan is critical for the inhibition of the LEDGF/p75–IN interaction (**Table 2**).

Next, we confirmed the mechanism of action of the CPs by performing STD NMR analysis for CP64, CP65, and their respective mutants. The STD NMR experiments used selective irradiation of well-separated protein signals at –1 ppm. Upon irradiation of target macromolecules, signals from the interacting ligands are enhanced by the intermolecular Nuclear overhauser enhancement phenomenon as illustrated in **Supplementary Figure S1**. The results of the STD NMR analysis presented in **Figure 2** demonstrate that both CP64 and CP65 bind to LEDGF/p75 but not at all or only very weakly to HIV IN. Since we used 40-fold excess of ligand relative to the target, such a weak interaction could be attributed to nonspecific binding of the peptides to IN. CP64m and CP65m bind neither to LEDGF/p75 nor to IN corroborating the importance of the tryptophan residue in the CPs to bind to LEDGF/p75 (**Figure 2**). A close examination of the STD NMR analysis of the LEDGF/p75-CP64 complex allowed us to determine the residues of CP64 that are in close contact with LEDGF/p75. Notably, very efficient saturation transfer occurred at the signals of the tryptophan and histidine side chains. Appreciable STD enhancements were also observed for backbone amide signals of leucine, tryptophan, and histidine. These STD NMR results corroborate the phage display strategy to identify peptides binding specifically to LEDGF/p75 and not to IN.

Table 1 Overview of the LEDGF/p75-binding peptides identified by phage display

Group	Peptide name[a]	Peptide sequence	Times selected[b]
Linear 7-mer		YPWLQSY	6
		LSTTLPL	1
Cyclic 7-mer	CP63	CVMGHPLWC[c]	4
	CP65	CILGHSDWC	3
	CP64	CVSGHPLWC	2
		CVEGHPAWC	1
		CVSGHPEFC	1
		CVMGHPTW**C**	1
		CVMGHPSWC	1
Linear 12-mer		FPWMQSYGVGIN	30

Abbreviation: LEDGF, lens epithelium-derived growth factor.
[a]Nomenclature of peptides selected for subsequent analysis. [b]Numbers indicate how often the peptide sequences were selected using $LEDGF_{325-530}$ as bait. [c]Peptides selected for further analysis are marked in gray.

Table 2 Structural and functional characterization of the peptides

Name	Sequence	LEDGF-IN IC_{50} (µmol/l)[a]	LEDGF-JPO2 IC_{50} (µmol/l)[a]	STD NMR[b]	Antiviral activity[c]
CP63	CVMGHPLWCGGGK[d]	19.37 ± 7.69	40.45 ± 19.39	n.d.[e]	+
CP64	CVSGHPLWCGGGK	35.88 ± 10.00	>200	LEDGF/p75	+
CP64m[f]	CVSGHPL**A**CGGGK	>200	n.d.	Negative	–
CP65	CILGHSDWCGGGK	59.89 ± 28.50	>200	LEDGF/p75	+
CP65m[f]	CILGHSD**A**CGGGK	>200	n.d	Negative	–
Scrambled peptides[g]					
CP65s[1]	CHISDLG<u>W</u>CGGGK	n.d.	n.d	n.d	+
CP65s[2]	CHSGDWLICGGGK	n.d	n.d	n.d	–

Abbreviations: ELISA, enzyme-linked immunosorbent assay; LEDGF, lens epithelium-derived growth factor; STD, Saturation transfer difference.
Cyclization of the peptides was done by a disulfide bridge of the cysteine residues at position 1 and 9.
[a]Mean values ± s.d. of 50% inhibitory concentration as determined by AlphaScreen from at least two independent experiments. [b]STD NMR analysis for target protein. If the peptides bind neither to LEDGF/p75 nor IN, the result is indicated as "negative." [c]Antiviral activity as determined by p24 ELISA in the supernatant. If the peptide inhibits viral breakthrough indicated as (+), otherwise indicated as (–). [d]The linker sequence is highlighted in gray. [e]Not determined. [f]Peptides with W to A substitution indicated in bold in the peptide sequence. [g]Scrambled peptide with a conserved tryptophan (underlined) position in the common motif (CP65s[1]) or a random scrambled peptide (CP65s[2]) were generated from CP65 and used in the cell-based antiviral assays.

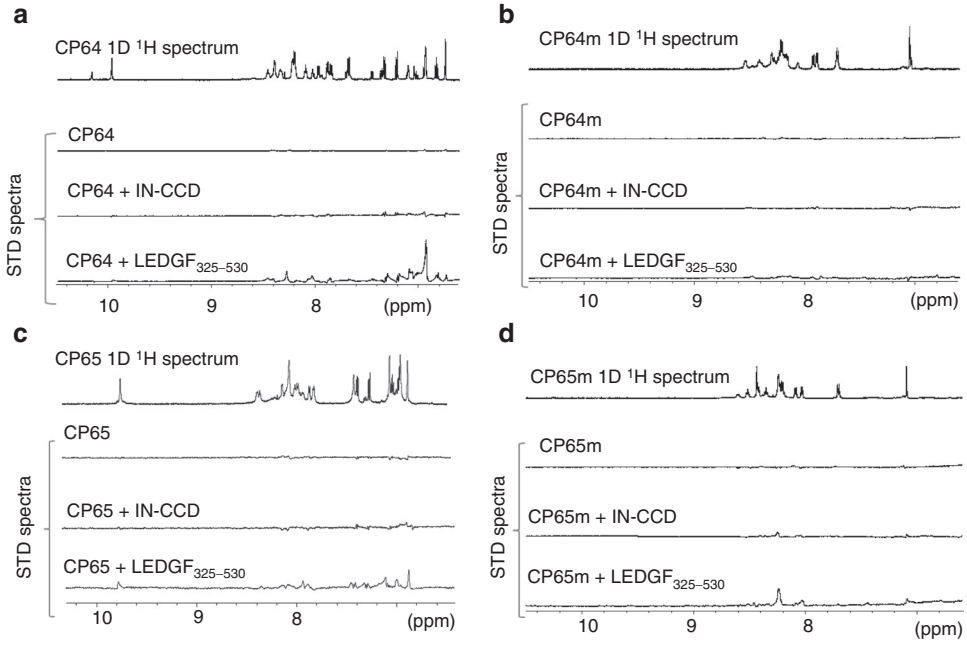

Figure 2 Saturation transfer difference (STD) NMR analysis for the lens epithelium-derived growth factor (LEDGF)/p75 interacting cyclic peptides (CPs). The recorded STD spectra plotted for each CP. At the top, a reference 1D ^{1}H NMR spectrum is shown for each CP displaying very narrow resonances typical for short peptides. A corresponding control STD NMR experiment with peptides only showed no signal indicating that the impurity contained in the spectrum can effectively be subtracted and therefore does not give rise to false positive signals in the difference spectrum obtained in the presence of the target proteins. STD NMR spectra for (**a**) CP64 and (**c**) CP65 showed specific signals only in the presence of $LEDGF_{325–530}$ but not with HIV-1 integrase (IN) catalytic core domain (CCD) demonstrating that CPs bind specifically to $LEDGF_{325–530}$. In contrast, (**b**) CP64m and (**d**) CP65m bind neither to $LEDGF_{325–530}$ nor to IN CCD. HIV, human immunodeficiency virus.

Anti-HIV activity of peptides with a charged transduction domain is primarily due to a block of viral entry

Intracellular delivery of biological cargoes including therapeutic peptides is often mediated by conjugating them with cationic cell-penetrating peptides (CPP).[18,19] Although, we have not systematically attempted to select the optimal CPP sequence among the different CPPs described in literature, CPPs have different translocation mechanisms.[18,19] We selected a well-described arginine-rich conjugate.[20] We examined the antiviral activity of CP63 and CP64 fused to the arginine-rich transduction domain. Both CP63 and CP64 inhibited HIV-$1_{NL4.3}$ replication in MT-4 cells with EC_{50} values in the low micromolar range (**Supplementary Figure S2a**). However, several arginine-rich peptides including HIV-1 Tat-derived CPP are known to interact with CXCR4 and thereby interfere with the entry process of HIV (for a review, see ref. 21). Notably, a virus strain resistant against the bicyclams AMD3100, a CXCR4 chemokine antagonist that blocks HIV-1 entry, was previously found to show cross-resistance against cationic inhibitors.[22,23] To exclude this potential artifact from our analysis we tested whether CP63 and CP64 would retain activity against AMD3100 resistant strain, HIV-$1_{NL4.3+AMD3100}$.[24] Since both CPs lost their antiviral activity, the observed inhibition by CP63 and CP64 of HIV-$1_{NL4.3}$ replication is probably due to a nonspecific charge-mediated virus entry block rather than through interference with LEDGF/p75–IN interaction (**Supplementary Figure S2a**). Moreover, we also observed that other reported anti-HIV cell penetrating cationic peptides failed to inhibit HIV-$1_{NL4.3+AMD3100}$ replication (F. Christ and Z. Debyser, unpublished results). Therefore, we believe that interference with the entry process by the cationic CPPs masks the real antiviral mechanism of action of peptides selected to interfere with intracellular processes. Different vehicles such as integrating vectors, peptide transfection using Plusin reagent or liposomes can be employed to deliver peptides into cells.[25] To obtain stable intracellular expression of peptides, we opted to use integrating lentiviral vectors expressing the peptides fused to monomeric red fluorescent protein (mRFP). Moreover, stable expression allows to measure the antiviral effect during multiple round replication and resistance selection. Previously, Dietz *et al.*[26] have shown that constitutive expression of small peptides fused to RFP inhibits the HIV-1 RNA packaging signal Ψ, indicating that fluorescent protein tagged peptides can be used to evaluate intracellular antiviral targets allowing easy monitoring of the stable expression of the peptides over time.

LEDGF/p75 interacting peptides do not interfere with the cellular functions of LEDGF/p75

The identified peptides in the original M13-derived phages were fused at the N-terminus of the peptide-displaying pIII capsid protein of the phages. We constructed the lentiviral vector expression cassettes accordingly by fusing each CP to mRFP (**Supplementary Figure S3a**). All fusion proteins (CP63; CP63-mRFP, CP64; CP64-mRFP, CP64m; CP64m-mRFP, CP65; CP65-mRFP, CP65m; CP65m-mRFP) were expressed to similar levels in HeLaP4 cells transduced with the respective integrating lentiviral vectors (**Supplementary Figure S3b**). First, we excluded an effect on the expression level of CD4, the cellular receptor for HIV (**Supplementary Figure S3c**). Furthermore, we observed no difference in the growth kinetics or viability among the different cell lines indicating that LEDGF/p75 ligands can be expressed in cells without toxicity (**Supplementary**

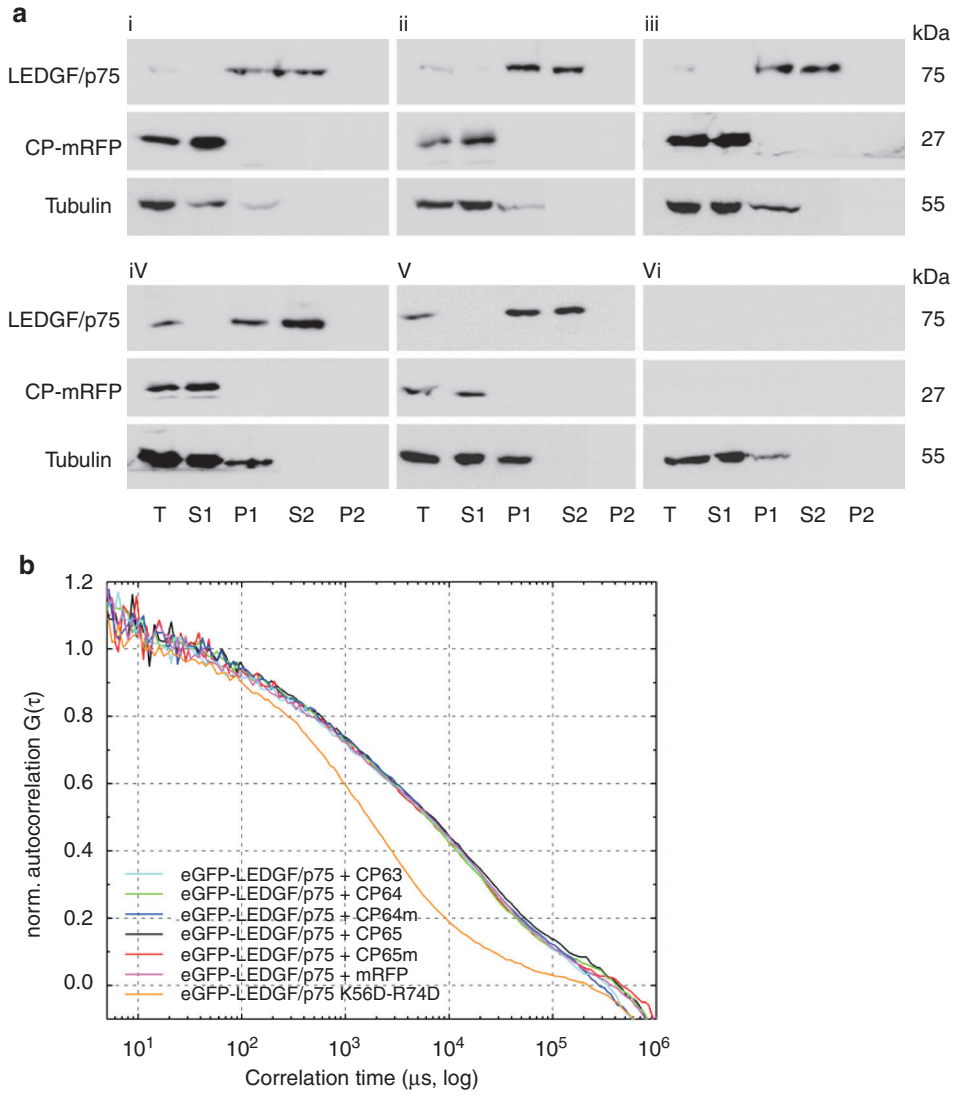

Figure 3 The cyclic peptides (CPs) do not interfere with cellular functions of lens epithelium-derived growth factor (LEDGF)/p75. (**a**) Chromatin-binding analysis performed as described before.[42,48] A representative western blot of the different cellular fractions is shown using antibodies against the indicated proteins. Extracts were made from (i) CP64-mRFP-, (ii) CP64m-mRFP-, (iii) CP65-mRFP-, (iv) CP65m-mRFP-, (v) mRFP-expressing cells, or (vi) the A3 (LEDGF/p75 depleted[47]) cell line. T, total cell lysate; S1, Triton-soluble cellular fraction; P1, Triton-insoluble cellular fraction; S2, DNase/$(NH_4)_2SO_4$-soluble cellular fraction; P2, DNase/$(NH_4)_2SO_4$-insoluble cellular fraction. (**b**) Fluorescence correlation spectroscopy (FCS) experiment with transiently expressed wild-type (WT) eGFP-LEDGF/p75 in each of the different CP-mRFP cell lines. The autocorrelation plots reveal that only the PWWP double-mutant K56D-R74D (eGFP-LEDGF/p75 K56D-R74D), displays impaired chromatin binding and scanning as described before.[48] Data represent mean values of five independent measurements for each condition. GFP, green fluorescent protein.

Figure S3d). To explore further and exclude potential side-effects of the CPs on the cellular function of LEDGF/p75, we performed chromatin binding assay and fluorescence correlation spectroscopy (FCS) analysis. The data shown in **Figure 3** and **Supplementary Figure S3e** substantiate that CPs can be stably expressed in eukaryotic cells without interference with the chromatin binding and scanning function of LEDGF/p75.

LEDGF/p75 interacting peptides inhibit HIV replication in cell culture

To probe the antiviral activity of the peptides against HIV-1, all peptides fused with mRFP were individually expressed in HeLaP4 cells and assayed for antiviral activities. The cells were infected with NL4.3 virus and viral replication was monitored by quantifying the HIV-1 p24 antigen in the supernatant over successive days. Cells expressing CP63, CP64, and CP65 resisted HIV-$1_{NL4.3}$ infection much longer than the control cells (**Figure 4a** and **Supplementary Figure S4**). This effect remained for more than two weeks indicating that the cells stably produce functional peptides even though fused to a larger fluorescent protein (**Supplementary Figure S4**). To confirm the specificity of the inhibitory effect of CP64 and CP65 against HIV-$1_{NL4.3}$, we tested cells expressing the inactive CPs (CP64m, CP65m) as well as mRFP alone. As expected, cells expressing CP64m or CP65m peptides failed to block viral breakthrough (**Supplementary Figure S4**). In the control cells, the HIV p24 protein level steadily increased during the first 10 days postinfection. The reduction in the amount of p24 protein in the supernatant at the later time points is due to cell death.

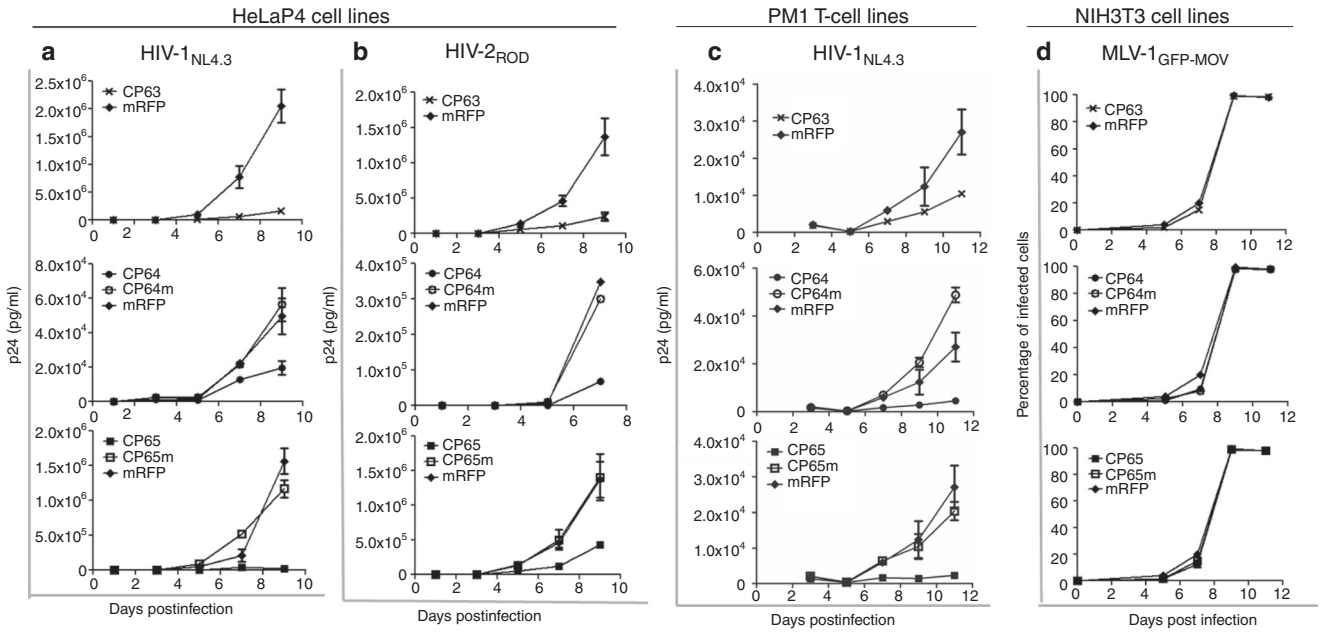

Figure 4 Antiviral activity of the lens epithelium-derived growth factor (LEDGF)/p75 interacting cyclic peptides (CPs). (**a**,**b**) HeLaP4 cells stably expressing CP63-, CP64-, CP64m-, CP65-, CP65m-mRFP, or mRFP alone were infected with (**a**) HIV-$1_{NL4.3}$ or (**b**) HIV-2_{ROD} and the p24 level was quantified in the supernatant at the indicated time points. (**c**) Similar experiments with HIV-$1_{NL4.3}$ were performed in PM1 cells expressing the above peptides and p24 was measured in the supernatant at the indicated time points. (**d**) NIH3T3 cells stably expressing the above fusion proteins were challenged with replication competent $MLV_{GFP-MOV}$ strain and green fluorescent protein (GFP) expression was monitored by fluorescent-activated cell sorter (FACS) at the indicated time points. HIV, human immunodeficiency virus.

Subsequent analyses of the antiviral activity of the peptides were done during the first 10 to 12 days when the p24 level peaks in the supernatant. We tested the spectrum of antiviral activity of the peptides by challenging the HeLaP4 cell lines that stably express CP63-, CP64- or CP65-mRFP with either HIV-$1_{NL4.3}$ or HIV-2_{ROD}. All cell lines resisted infection by HIV-1 or HIV-2 much longer than cells expressing either the respective inactive peptides or mRFP alone (**Figure 4a,b**). In contrast to the synthetic peptides with the transduction domain, cells constitutively expressing CP63, CP64, and CP65 inhibited HIV-$1_{NL4.3+AMD3100}$ breakthrough to the same extent as for the wild-type strain (**Supplementary Figure S2**). We also expressed the peptides in PM1 cells, a human T cell line commonly used for anti-HIV assays. At first, we characterized the cell lines for the expression of the fusion proteins and CD4 level, and growth kinetics as shown in **Supplementary Figure S5**. Subsequently, we infected the different cell lines including the controls with HIV-$1_{NL4.3}$ normalized for p24 level. All the active peptides blocked viral replication. As expected, viral breakthrough was unaffected in cells expressing the inactive peptides (CP64m, CP65m) and in control cell lines (**Figure 4c**).

We next analyzed whether the antiviral activity of the CPs correlates with the specific role of LEDGF/p75 during lentiviral replication. Previously, others and we have showed that LEDGF/p75 supports lentiviral DNA integration but not that of other retroviruses.[27,28] We tested whether the replication of murine leukemia virus (MLV), a gammaretrovirus, which is independent of LEDGF/p75–IN interaction but requires integration into the host chromatin,[27,28] could be inhibited by CP63, CP64, or CP65 stably expressed in NIH3T3 cells (**Supplementary Figure S5**). Subsequently cells were infected with replication competent green fluorescent protein (GFP)-encoding MLV[29] and viral breakthrough was evaluated by fluorescence-activated cell sorter (FACS) analysis for cumulative GFP expression. Interestingly, MLV infection was not inhibited by the LEDGF/p75 interacting peptides (**Figure 4d**), corroborating the specificity of the observed inhibition.

Since the selected CPs have a common motif (V^M/xGHP^L/$_X$W) with a particular role of the tryptophan at the C-terminus which was found to interact intimately with LEDGF/p75 in the STD NMR analysis (**Figure 2**), we wondered if scrambling the peptide sequences would affect their antiviral activity. To test this hypothesis we scrambled the CP65 peptide either by keeping only the tryptophan at its original position (CP65s^1) or by randomly changing the sequence of the peptide to abolish the original common motif identified in the phage display (CP65s^2) (**Table 2**). We then challenged the HeLaP4 cell lines that stably express the individual peptides CP64-, CP65-, CP65m-, CP65s^1-, CP65s^2-mRFP, or mRFP alone with HIV-$1_{NL4.3}$ (**Supplementary Figure S5**). CP64, CP65, and CP65s^1 (with the conserved tryptophan residue) resisted viral breakthrough (**Figure 5**). However, cells that express either the inactive peptide (CP65m), scrambled peptide (CP65s^2) or mRFP alone were susceptible to infection by HIV-$1_{NL4.3}$ (**Figure 5**). This result corroborates the specificity of the antiviral activity of the CPs and the necessity of the tryptophan residue of the peptides to bind to LEDGF/p75 and disrupt its interaction with HIV IN.

Mechanism of action of LEDGF/p75 interacting peptide ligands

To assess the molecular mechanism of the antiviral activity of the LEDGF/p75 interacting peptides, we first performed cross-resistance profiling. CP64 and CP65 inhibited the replication of

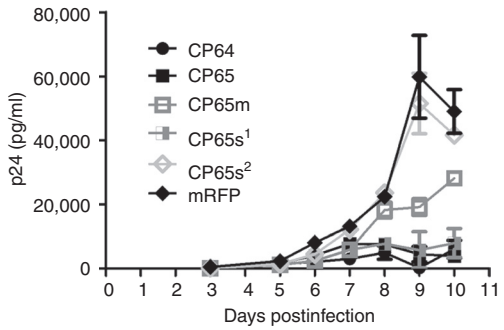

Figure 5 The antiviral activity of lens epithelium-derived growth factor (LEDGF)/p75 interacting peptides is dependent on the conserved tryptophan residue. The antiviral activity of the peptides is dependent on the tryptophan residue and its orientation in the common motif. A scrambled peptide with conserved tryptophan position (CP65s[1]) retains its antiviral activity while the random scrambled peptide (CP65s[2]) lost its activity completely as evaluated by NL4.3 viral breakthrough in HeLaP4 cells expressing CP64-, CP65-, CP65m-, CP65s[1]-, CP65s[2]-mRFP, or mRFP alone. Mean values ± s.d. from at least duplicate measurements of representative experiment is shown.

HIV-1 strains resistant to raltegravir[30] (**Figure 6a,b**) and a strain selected to be resistant against transdominant inhibition by the IBD[9] (**Figure 6c**). Whereas the IBD-resistant strain carrying mutations A128T/E170G was completely insensitive to LEDGINs,[10] both CP64 and CP65 blocked its replication in accord with their distinct mechanism of action (**Figure 6c**). It follows that the HIV-$1_{A128T/E170G}$ strain which is still dependent on LEDGF/p75[9] for replication is inhibited by CP64 and CP65.

Next, we tested whether CP64 and CP65 block HIV integration using quantitative PCR. Zidovudine (AZT) and raltegravir were included as reverse transcription and integration inhibitor controls, respectively. In agreement with the described role of LEDGF/p75 in lentiviral integration,[5] CP64 and CP65 inhibited HIV integration without significantly affecting reverse transcription (**Figure 6d**). However, the effect of CP64 and CP65 on HIV integration was less pronounced. This modest effect of the CPs on the number of integrated copies of HIV-1 was further reflected by the 2-LTR circle analysis where no significant increase was detected (data not shown). This may be due to the relatively lower potency of the CPs or to additional inhibitory effects of the peptides later in the replication cycle.

Therefore, we evaluated the impact of the LEDGF/p75-interacting peptides on both the production efficiency and the infectivity of progeny virions. Virus production was measured after transfecting cells expressing CP64 and CP65 peptides with HIV-$1_{NL4.3}$ proviral plasmid, bypassing the early stages of the replication cycle. As a result, only inhibitors targeting the later stages of viral replication (i.e., post-integration) can inhibit production of mature virus. The IN inhibitor raltegravir and the protease inhibitor ritonavir were included as early and late step inhibitor controls, respectively. Neither the CPs nor raltegravir affected the amount of HIV-$1_{NL4.3}$ produced in comparison with the control (mRFP) cells (**Figure 6e**). As expected, ritonavir significantly reduced virus production as quantified by p24 ELISA that measures only the processed capsid protein. Not surprisingly, viral particles produced in the presence of ritonavir profoundly lost infectivity due to the known maturation defect (**Figure 6f**). Raltegravir on the other hand, did not affect the infectivity of HIV-$1_{NL4.3}$ when added during production. Interestingly, CP65 also reduced the infectivity of the newly produced progeny virions (**Figure 6f**). Possibly, newly produced HIV-1 virions might require LEDGF/p75-IN interaction for reasons that are yet to be determined.

Failure to select resistance against LEDGF/p75-interacting peptide ligands

Resistance selection against inhibitors targeting virus-encoded proteins, even in the context of PPIs, appears fairly quickly.[9,10,31] For instance, when passaging HIV in the presence of increasing concentrations of LEDGIN 6, genotypically resistant virus with a single amino acid substitution in IN (IN A128T) was detected as early as 4 weeks and full conversion was obtained at week 20.[10] Moreover, resistance selection against the transdominant inhibition of the IBD of LEDGF/p75 resulted in the selection of the IN A128T/E170G strain at around 5 weeks.[9] However, it remained to be seen whether resistance against inhibitors that target the host partner could appear as readily. We therefore attempted to select resistance by serial passaging of HIV-$1_{NL4.3}$ in HeLaP4 cells expressing CP65-mRFP. As a no-inhibitor control, we passaged the virus in parallel in CP65m-mRFP and mRFP expressing cells to ensure virus growth in the absence of inhibitor. Cytopathic effect induced by the virus was examined periodically to monitor virus growth as a guide for passaging. The selection pressure was progressively increased during the experiment by reducing the volume of the progeny virus inoculum as described before.[9] Although, we continued resistance selection for over 100 days, no mutations were detected in the *RT* and *IN* genes of the virus.

DISCUSSION

In recent years, insight into host-pathogen PPIs and their networks has increased dramatically, fueling the impetus to develop novel therapeutics.[32] This is particularly tempting when targeting the host protein since its inherent low genetic variability is expected to increase the genetic barrier to resistance development. On the other hand, one should carefully avoid cellular toxicity associated with the interference with the physiological functions of the host protein.

Viral integration is a particularly attractive target because it is an essential step during HIV replication. Integration of the reverse transcribed cDNA of the lentiviral RNA genome into the host chromatin is mediated through an organized multistep process orchestrated by the viral IN.[33] In search of novel classes of antiretrovirals, recently an array of small molecule HIV IN inhibitors that block the integration reaction by binding to the catalytic site or to noncatalytic sites of IN have been reported. These compounds are at different stages of development. Raltegravir is to date the only FDA-approved IN strand transfer inhibitor available for patient treatment (for an extensive review, see ref. 34). By virtue of its central roles in HIV replication, the LEDGF/p75–IN interaction is widely considered as an attractive and novel antiviral target.[4,35] We recently discovered LEDGINs as small molecules that bind to the LEDGF/p75 binding pocket in IN and potently inhibit HIV-1 replication.[10] LEDGINs provide proof-of-concept that allosteric inhibitors of integration targeting virus–host interaction might become an integral part of highly active antiretroviral therapy in the future.[10]

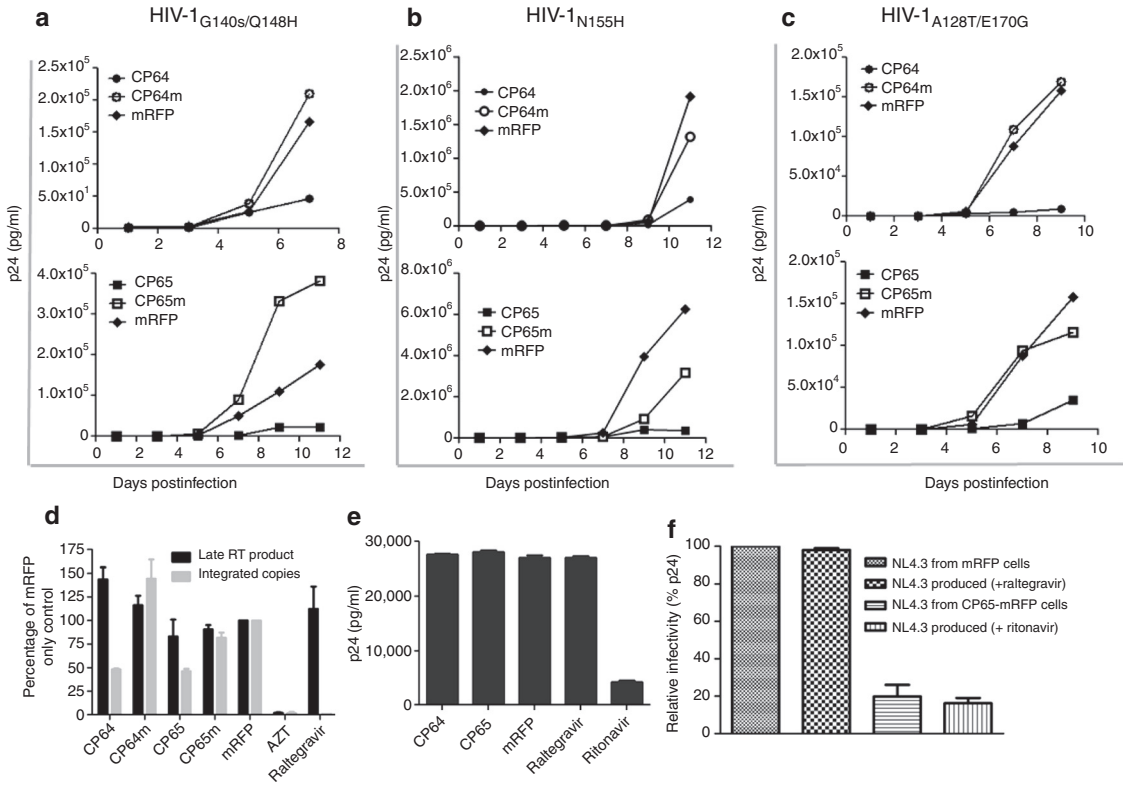

Figure 6 Mechanism of action studies for CP64 and CP65. (**a–c**) Show the cross-resistance profile of CP64 and CP65. Activity against raltegravir resistant strains, (**a**) HIV-$1_{G140S/Q148H}$[30] and (**b**) HIV-1_{N155H}.[30] (**c**) Antiviral activity against a strain resistant to transdominant inhibition by IBD, HIV-$1_{A128T/E170G}$.[9] (**d**) Human immunodeficiency virus (HIV) DNA quantification by quantitative PCR (qPCR) analysis to determine the effect of cyclic peptides (CPs) on the levels of late reverse transcriptase (RT) product (total HIV DNA) at 10 hours postinfection. Integrated copies were quantified after 7 days to ensure that only the integrated DNA species was measured. The data is presented as percentage with respect to cells expressing only mRFP (control). AZT and raltegravir are included as controls for inhibition of RT and integration. (**e**) Effect of CPs on virus production from the NL4.3 plasmid transfected in HeLaP4 cells that express the respective peptides as quantified by p24 enzyme-linked immunosorbent assay (ELISA). Raltegravir and ritonavir were included as early and late stage inhibitor controls. (**f**) Lens epithelium-derived growth factor (LEDGF)/p75 interacting CPs significantly reduces the infectivity of viral progeny. 48 hours post-transfection, the supernatants from cells expressing CP65 or mRFP were transferred to HeLaP4 cells expressing the mRFP alone (control) and the amount of p24 was quantified 72 hours postinfection. As control, NL4.3 was produced in HeLaP4 cells expressing mRFP in the presence of either raltegravir (30 nmol/l) or ritonavir (300 nmol/l) and the infectivity of the virus was assessed in HeLaP4 control cells. Data represent mean values ± s.d. from three independent experiments.

In line with this, we have now taken the reciprocal approach targeting LEDGF/p75 rather than HIV-1 IN. We employed the C-terminal fragment of LEDGF/p75 containing the IBD as bait for screening of three different phage libraries and identified peptides that block LEDGF/p75-IN interaction *in vitro* and inhibit HIV replication. We focused on the IBD of LEDGF/p75 because it is the principal interacting domain with HIV IN and recent studies have shown that it is also an important domain for several other PPIs [7,17,36,37] making it an appealing and versatile drug target. These peptides are expected to be less toxic as they do not interfere with the chromatin binding function of LEDGF/p75 (**Figure 3**) nor bind to its splice variant, LEDGF/p52.[38] Nevertheless, the selectivity for a particular LEDGF/p75 interaction may be of a concern since the peptides were identified from combinatorial libraries of phages. Our results suggest, however, that selectivity is achievable (at least among the tested CPs), as CP64 and CP65 preferentially inhibit the LEDGF/p75–IN interaction *in vitro* and block HIV replication at nontoxic levels in cells. Although, lentiviral IN, MLL1/menin complex, JPO2 and PogZ have overlapping binding sites on LEDGF/p75, the detailed architecture of the interaction differs.[7,17,36,37] An important remaining task is to improve the affinity of these peptides and define the binding site on LEDGF/p75 by co-crystallization. Moreover, our data fuel the search for small molecules or peptidomimetics binding to LEDGF/p75 with increased potency and druggability.

Although identification of peptide-based inhibitors is an important aspect of drug discovery, their physicochemical properties hamper their delivery into cells.[39] In spite of this limitation, CPs that modulate PPIs help to validate drug targets. Stable expression of peptides fused to large proteins in cells is a useful tool to explore the biological effects of inhibitors. The data from present as well as previous studies indicate that stable expression of peptides targeting intracellular proteins can inhibit HIV replication suggesting that fusing the peptides to large fluorescent proteins does not abolish or bias their antiviral activity.[26,40]

Most of the CPs share a common motif with a specific orientation of the tryptophan residue influencing the antiviral activity (**Figure 4–6**). The common peptide motif with minimal variation suggests that the peptide-binding site on LEDGF/p75 is a conserved region, which may be targeted as well by small molecules

or peptidomimetics. Interestingly, apart from the CPs, both frequently isolated linear peptides share a common motif (**Table 1**). Although, we selected the CPs because of their inherent stability to perform a thorough analysis and validate LEDGF/p75 as antiviral target, the linear peptides may require further evaluation. It will be interesting in future drug discovery efforts to design peptidomimetics based on the minimal common motif of both the cyclic and the linear peptides. Venkataraman *et al.*[41] reported that different human cells are capable of synthesizing mature CPs that block HIV replication. Although, we do not know whether our peptides are cyclic or not in cells as it is impossible to assess the cyclic nature of peptides expressed as fusion proteins, the specific antiviral activity of the peptides suggests that the CPs are probably cyclic in the oxidizing environment of the cell.

The effect of the peptides on HIV integration is in agreement with previous reports where interference of the LEDGF/p75–IN interaction either by LEDGINs[10] or by depletion of LEDGF/p75[42] blocks integration into the host chromatin, albeit to a lesser extent. In addition to the effect on integration, we noticed that CP65 inhibited viral infectivity by approximately fivefold (**Figure 6f**) without significant effect on the number of viral particles produced (**Figure 6e**). This discrepancy suggests that the antiviral activity of the peptides is based on the cumulative effect suggesting that the LEDGF/p75–IN interaction may also be required during the later stages of HIV replication. How the CPs affect the infectivity of progeny virions remains to be elucidated. At this stage, we can only speculate that LEDGF/p75 may have a role during the later stages of HIV replication.[42]

Resistance development is a major concern in the development of new anti-HIV drugs particularly for those targeting virus encoded proteins. Given the extremely low rate of variability of cellular proteins, resistance related mutations are highly unlikely when the host counterpart of the virus-host PPIs is targeted. Our result for CP65 supports this notion. Considering the vulnerability of conventional antiretrovirals to resistance development, future ARV discovery could aim at identifying small molecules targeting cellular proteins in the context of virus-host PPIs.

In conclusion, we have demonstrated that screening of phage libraries can successfully identify peptides that bind to LEDGF/p75 and inhibit HIV replication. Moreover, our results validate LEDGF/p75 as a genuine antiviral target and provide essential templates for developing future therapeutics targeting this cofactor. Our approach supports the new paradigm in the field of antiviral research based on the inhibition of the interaction between viral proteins and intracellular cofactors. Because LEDGF/p75 is an important protein at the center of other human diseases such as leukemia,[7] it constitutes an appealing drug target for multiple unmet medical needs. Applications of the work presented here may thus well surpass the field of antiviral research.

MATERIALS AND METHODS

Phage display biopanning strategy. Paramagnetic beads (Dynabeads M-280 tosylactivated; Invitrogen, Carlsbad CA) were coated with purified recombinant proteins including wild-type HIV-1 IN, the C-terminal fragment of LEDGF/p75 ($LEDGF_{325–530}$) as well as various controls[8] according to the manufacturer's instructions. Dynabeads coated with HIV-1 IN with were washed with TBSGT [50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.25% (wt/vol) gelatin, 0.1% (vol/vol) Tween 20] and preincubated with 10 µl of the original M13 phage display libraries (New England Biolabs, Ipswich, MA) encoding random linear 7-mer, cyclic 7-mer, or linear 12-mer peptides in phosphate-buffered saline/0.25% gelatin (PBSG; Gibco-Invitrogen, Grand Island, NY; Fisher Scientific, Fair Lawn, NJ) while rotating at 4 °C overnight. Phages bound nonspecifically to the beads or IN, were discarded, the supernatant was used for the first positive selection with $LEDGF_{325–530}$ coated beads. The next day, beads were washed ten times with PBSG/0.5% (wt/vol) Tween-20 (PBSGT; Sigma-Aldrich, St Louis MO) and bound phages were eluted by pH shift with 0.2 mol/l glycine–HCl (pH 2.2) supplemented with 1 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich) and neutralized with 1 mol/l Tris, pH 9.1. Overall, we performed three rounds of positive selection and phages of the second and third positive elution were titered. Single clones were picked and tested in phage ELISA for binding to the individual target and control proteins. Clones binding specifically to the $LEDGF_{325–530}$ were individually amplified for 4.5 hours at 37 °C by infecting *Escherichia coli* ER2738 cells with the supernatant, phage DNA was prepared and the region of interest was sequenced to deduce the foreign peptide insert according to the manufacturer's manual with the supplied 96gIII primer.

Phage ELISA. Plates (Greiner-Bio-One, Frickenhausen, Germany) were coated overnight at 4 °C with 100 ng/well of wild-type IN or $LEDGF_{325–530}$ or BSA. The next day, plates were washed three times with dH_2O and blocked for 1 hour at room temperature with 200 µl/well of 3% milk powder (Sigma-Aldrich) in PBS/0.5% Tween-20 (PBSMT). Plates were washed three times with dH_2O in an automated plate washer (BioTek Instruments, Winooski, VT) and 70 µl supernatant of overnight amplified phages presenting $LEDGF_{325–530}$ binding peptides added to 30 µl of PBSMT were applied to the plates and incubated in parallel overnight at 4 °C. Plates were washed three times and incubated for 1 hour at room temperature with 100 µl/well of an anti-M13 HRP-conjugated antibody (1:2,000 in PBSMT; GE Healthcare Bio-Sciences, Piscataway. NJ). After washing five times, the plates were developed with 100 µl/well of *o*-phenylenediamine in phosphate-citrate buffer (Sigma-Aldrich), stopped with 100 µl/well of 1 N H_2SO_4 (VWR, West Chester PA) and read at 490/620 nm to detect bound phages.

Peptide analysis. Phage inserts were analyzed for linear and conformational homology using a computer program (3DEX) described elsewhere.[15] Sequences were checked against linear homology to the protein sequence of target protein as well as to available structures (PDB entry 1HYV[43] and 1Z9E[44]). Clones were grouped according to their peptide motifs and the library used.

AlphaScreen and integration assay. The selected CPs were synthesized, head-to-tail cyclized through disulfide bond and purified (>90% purity) by Pepscan Systems (Leiden, the Netherlands) and EZBiolab (Carmel, IN). His_6-HIV-1 IN, flag-LEDGF/p75, and MBP-JPO2 were purified for AlphaScreen assays as described previously.[17] The AlphaScreen assay was performed according to the manufacturer's protocol (Perkin Elmer, Benelux, Belgium). We performed the reactions in 25-µl final volume in 384-well Optiwell microtiter plates (Perkin Elmer). The reaction buffer contained 25 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 0.01% (vol/vol) Tween-20 and 0.1% (wt/vol) BSA. His_6-HIV-1 IN (300 nmol/l final concentration) was incubated with the peptides for 30 minutes at 4 °C. The peptides were added at varying concentrations spanning a wide range from 0.1 up to 100 µmol/l. Then, 100 nmol/l flag-LEDGF/p75 was added, and incubation was prolonged for an additional hour at 4 °C. Subsequently, 5 µl of Ni-chelate-coated acceptor beads and 5-µl anti-flag donor beads were added to a final concentration of 20 µg/ml of each. Proteins and beads were incubated for 1 hour at 30 °C in order to allow association to occur. Exposure of the reaction to direct light was omitted as much as possible and the emission of light from the acceptor beads was measured by the EnVision plate reader (Perkin Elmer). Data was analyzed using the EnVision manager software. Counter screens with

JPO2 were essentially performed as described previously.[10,17] Both IN overall as well as strand transfer enzymatic reactions were carried out as described previously.[10]

STD NMR spectroscopy. All NMR experiments were performed in the same buffer conditions as the AlphaScreen assay but omitting BSA (25 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 0.01% (vol/vol) Tween-20 and 0.1% (wt/vol). Spectra were acquired on a Bruker 600-MHz spectrometer 5 mm TXI HCN Z gradient cryoprobe (BioMacS; KULeuven, Leuven, Belgium) at 22 °C. The MBP-$LEDGF_{325-530}$ or IN-CCD at 50 µmol/l was incubated with different CP at 2 mmol/l. One dimensional proton spectra were acquired as a reference using excitation sculpting to suppress the water signal. In the STD NMR experiments, a 2 seconds saturation on the F2 channel was alternated between −1 and 20 ppm to obtain a saturation transfer spectrum and reference spectrum, respectively. For each spectrum, 256 scans were acquired, the water signal was suppressed using excitation sculpting and a spinlock was used to suppress protein signals.[45] The saturation transfer spectrum was subtracted from the reference spectrum to obtain a final difference spectrum. The control STD NMR experiments with on- and off-resonance frequencies set at −1 and 20 ppm, using a saturation time of 2 s, were carried out on isolated CP at 2 mmol/l concentration. No signals were present in the difference spectra, indicating that the effects observed in the presence of LEDGF/p75 resulted from a true saturation transfer.

Oligonucleotide cloning and lentiviral vectors. The oligonucleotides for cloning purpose were designed based on the sequences of the peptides selected by phage display modified with addition of a start codon, cysteine residues at both ends and an extra C-terminal flexible four amino acids linker sequence (**Tables 1** and **2**). Each amino acid was converted to the corresponding codon based on the preference obtained from the human codon usage table. All precautions were taken to avoid codon bias in human cell lines while expressing the oligonucleotides as fusion proteins. For the sequences of all the oligonucleotides and primers used in this study, see **Supplementary Table S3**.

We cloned the different CPs as mRFP fusion proteins in the pmRFP-N_1 expression plasmid. After knocking out the start codon (ATG to ATT) of mRFP using the site-directed PCR mutagenesis method, the annealed CP oligonucleotides were ligated in frame at the N-terminus of mRFP. The presence of the expected fusion proteins was confirmed by DNA sequencing of the entire CP-mRFP fusion-coding region ensuring that no additional mutations were present in the molecular clones. To construct the lentiviral transfer plasmids pCombi-CP-mRFP-IRES-Puro, the fragment encoding eGFP was removed from the plasmid pCombi-eGFP-IRES-Puro by *BamH*I and *Spe*I digestion and replaced by the *Bgl*II-CP-mRFP-*Xba*I fragment from the pPeptide-mRFP-N1 plasmids encoding the peptide-mRFP fusion proteins. Whereas for NIH3T3 and T-cell lines expression, the pSFFV-CP-mRFP-IRES-tCD34 was constructed by removing eGFP encoding fragment from pSFFV-eGFP-IRES-tCD34 by *Xba*I and *Xho*I digestion and replaced by a PCR fragment of *Xba*I-CP-mRFP-*Sal*I from the respective lentiviral vector plasmids generated above. Production of lentiviral vectors encoding the different CPs was performed as described previously by Geraerts *et al.*[46]

Cell culture and generation of stable cell lines. The HEK293T and NIH3T3 cells maintained in DMEM (GIBCO BRL, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS; Harlan Sera-Lab, Loughborough, UK), and 50 µg/ml gentamicin (GIBCO BRL). HeLaP4 cells were grown in DMEM containing 10% FCS, 50 µg/ml gentamicin, and 0.5 mg/ml geneticin (GIBCO BRL) from here on referred as complete DMEM. A3 cells, LEDGF/p75 depleted monoclonal HeLaP4-CCR5 cells[47] were maintained in DMEM containing 10% FCS, 50 µg/ml gentamicin, 0.5 mg/ml geneticin and 200 µg/ml zeocin (Invitrogen, Merelbeke, Belgium). The PM1 T-cell lines, a kind gift from Dorothee von Laer (Innsbruck Medical University, Innsbruck, Austria), were maintained in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS, 50 µg/ml gentamicin. All cell lines were grown in a humidified atmosphere with 5% CO_2 at 37 °C.

To generate polyclonal cell lines stably expressing the fusion proteins, 50,000 cells/well were seeded in a 24-well plate. The following day, cells were transduced overnight with the lentiviral vectors encoding for: CP63-mRFP, CP64-mRFP, CP64m-mRFP, CP65-mRFP, CP65m-mRFP, CP65s[1]-mRFP, CP65s[2]-mRFP, or mRFP to generate the respective HeLaP4 cell lines. After 72 hours, selection was initiated by adding 2 µg/ml puromycin (Invitrogen). To generate individual CP encoding NIH3T3 or PM1 cell lines, we used the SFFV-CP-mRFP-IRES-tCD34 lentiviral vectors expressing the CP-mRFP fusion protein. Their expression level was verified by western blotting and FACS analysis.

Cell growth assay. Five thousand cells per well of the different adherent cell lines (HeLaP4, A3 or NIH3T3) encoding CPs were plated in 12-well plates; for the PM1 cell lines, 50,000 cells per well in 48-well plate were seeded and cells were counted on successive days.

Western blotting and chromatin binding assay. Western blotting, cellular fractionation, and chromatin-binding assays were performed as described before.[42] Proteins were probed overnight at 4 °C with either rabbit anti-LEDGF/p75 (1:1,000; Bethyl Laboratories, Montgomery, TX) or rabbit anti-mRFP antibody (1:500; Chemicon, Temecula, CA). Visualization was performed using chemiluminescence (ECL+; Amersham Biosciences, Uppsala, Sweden) after incubating for 2 hours at room temperature with anti-rabbit antibody coupled to horseradish peroxidase (Dako, Glostrup, Denmark).

Fluorescence correlation spectroscopy (FCS). Fluorescence correlation spectroscopy (FCS) experiments were performed as described before.[48] In short, HeLaP4 cells were transiently cotransfected with two plasmids encoding for eGFP-LEDGF/p75 and peptide-mRFP. Absolute protein concentrations and chromatin scanning of eGFP-LEDGF/p75 was measured in living cell nuclei. To specifically probe the effect of the peptides on the chromatin binding and scanning function of LEDGF/p75, transfections were optimized. Measurements were only performed in cells containing a 20–50-fold molar excess of the peptide-mRFP. As a control for impaired chromatin binding and scanning properties of LEDGF/p75, we included the K56D-R74D double mutant in the PWWP domain of LEDGF/p75 described previously.[48] Data represent mean values of five independent measurements for each condition.

Confocal laser scanning microscopy. HeLaP4 cells stably encoding the various peptide-mRFPs were immunostained with the LEDGF/p75 antibody. Confocal laser scanning microscopy was performed on a Zeiss LSM510 system (Carl Zeiss, Jena, Germany) as described before.[47]

Flow cytometeric analysis. Briefly, 1×10^6 cells of the different cell lines were pelleted, washed once with PBS and incubated at room temperature with 10 µl of mouse FITC-conjugated monoclonal antihuman CD4 antibody (Clone Q4120; Sigma-Aldrich, St Louis, MO) in 100 µl PBS in the dark for 30 minutes. The cells were washed once with PBS and then fixed with 2% paraformaldehyde. Analysis of the cell surface CD4 receptor expression of the different CPs-encoding cells was performed by a FACS, Becton Dickenson FACS (Calibur). To verify the transduction efficiency of the fusion proteins, we used the expression of mRFP for HeLaP4 or we stained for CD34 with an antihuman CD34 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) for PM1 cell lines encoding the various CPs, and the FACS analysis was performed as mentioned above.

Virus strains. The HIV-1 molecular clone, NL4.3 and the HIV-2 molecular clone, ROD are described before.[10] The MLV molecular clone, GFP-MOV,[29] is a replication-competent ecotropic MLV variant expressing the chimeric GFP-Env protein and is a kind gift of B. Schnierle (Paul-Ehrlich Institut, Langen, Germany). The HIV-1 molecular clones: HIV-$1_{NL4.3+AMD3100}$,[24] HIV-$1_{G140S/Q148H}$,[30] HIV-1_{N155H},[30] and HIV-$1_{A128T/E170G}$[9] were described previously.

HIV infection. The HeLaP4 cell lines encoding CPs were seeded in parallel in 6-well plates at a cell density of 20,000/well. The following day, cells were

infected with the different HIV strains normalized for p24 levels in 2 ml of complete DMEM. The next day, the supernatants were removed; cells were washed twice with PBS and fresh 4 ml medium added. For PM1 T-cell lines expressing various CPs, 50,000 cells/well were seeded in 48-well plates and HIV infection was performed with inocula normalized for p24 level. To monitor the HIV replication and antiviral activity of the CPs, p24 level was determined in the supernatants using a commercial p24 ELISA kit (Innogenetics, Ghent, Belgium).

MLV infection. The NIH3T3 cell lines encoding each CP were seeded in 6-well plates at a cell density of 10,000/well and on the following day; cells were infected overnight with $MLV_{GFP\text{-}MOV}$. The next day, cells were washed twice with PBS and we added fresh medium for the cells. The viral breakthrough was monitored over 10 days by FACS analysis for cumulative GFP expression.

Real-time PCR quantification of HIV DNA. HeLaP4 cells stably encoding CP64, CP65, or control (mRFP) were seeded at 10^6 cells per well in a 6-well plate. After 24 hours they were infected with HIV-$1_{NL4.3}$ (equivalent of 2 µg of p24) in 2 ml DMEM for 3 hours. Subsequently washed three times with PBS and incubated with fresh medium in the presence of ritonavir (3 µmol/l). Ritonavir was added to block re-infection during the period of the experiment. Total DNA extraction was performed using the QIAamp blood kit (Qiagen, Benelux B.V., Belgium). Quantification of the viral DNA such as late RT product, 2-LTR circles and integrated copies was done as described before,[49] with modification of the primer-probe set to quantify the late RT as well as integrated copies since the peptide expressing cell lines were generated using HIV-based vectors. For this purpose, we designed a set of TaqMan probe and primers to quantify specifically HIV-1 DNA in the cell lysate. For the quantification of total HIV-1 DNA, the forward primer (RT-gag1): 5′-ATCAAGCAGCCATGCAAATGTT-3′, the reverse primer (RT-gag2): 5′-CTGAAGGGTACTAGTAGTTCCTGCTATGTC-3′ and the probe (RT-gag probe): 5′-(FAM)-GACCATCAATGAGGAAGCTGCAGAATGGGA-(TAMRA)-3′ were used. Samples were harvested at 10, 30, and 168 hours postinfection for the analysis of the late RT products, 2-LTR circles and integrated copies, respectively. While the late RT products and integrated copies were measured using the above primer-probe set designed to only detect viral DNA but not the integrated HIV-based vectors used to constitutively express the peptides, the 2-LTR circles were quantified using the primer-probe set described before.[49] AZT and raltegravir were included as RT and integration inhibitor controls.

Resistance selection. The resistance selection was performed as described before.[9] We initiated the selection in HeLaP4 cells expressing CP65-mRFP with HIV-$1_{NL4.3}$ at high multiplicity of infection (MOI = 10). Simultaneously, as noninhibitor controls, virus was also passaged in HeLaP4 cells expressing CP65m-mRFP or mRFP alone expressing cells. Every 5–6 days, the cell culture was monitored for the appearance of HIV-induced cytopathic effect (CPE). When CPE was observed, the cell-free culture supernatant was used to infect fresh, uninfected HeLaP4 cells expressing CP65-mRFP or control cells. To increase selective pressure, the volume of supernatant transferred was progressively decreased during the experiment from 1 ml to 10 µl for 3×10^4 cells cultured in 4 ml of medium.

PCR amplification and sequencing of the RT- and IN-coding regions. Proviral DNA extraction of infected CP65-mRFP or control (CP65m-mRFP and mRFP alone) cells was performed using the QIAamp blood kit (Qiagen). PCR amplification and sequencing of RT- and IN-encoding sequences were done as described previously.[9]

Transfection of pNL4.3 plasmid DNA in HeLaP4 CP encoding cells and analysis of the infectivity of the virus. The different CPs encoding and control cell lines (700,000 cells/well) were seeded in a 6-well plate. The following day, the medium was removed and cells were transfected with 1 µg pNL4.3 plasmid DNA using 3 µl Fugene-6 following the manufacturer's instructions. As controls, 30 nmol/l of raltegravir or 300 nmol/l of ritonavir were included. Twenty-four hours post-transfection, virus production was assessed by quantifying the p24 antigen in the supernatant. Cells were seeded at 50,000 cells per well in 24-well plates and infected with 50 ng of p24 equivalent virus in 500 µl for 4 hours and washed two times with PBS. Seventy two hours postinfection the amount of p24 protein in the supernatant was quantified.

SUPPLEMENTARY MATERIAL

Figure S1. Schematic illustration of the STD NMR analysis.
Figure S2. Intracellular expression of CPs provides better antiviral evaluation than using peptides fused to a transduction domain.
Figure S3. Characterization of HeLaP4 cell lines stably expressing the CPs.
Figure S4. Antiviral activity of the constitutively expressed CP64 and CP65 in HeLaP4 cells and the kinetics of viral breakthrough.
Figure S5. Characterization of different cell lines stably expressing the cyclic peptides (CPs).
Table S1. Results of phage ELISAs to detect the binding specificity of phages revealing the different peptides for $LEDGF_{325\text{-}530}$.
Table S2. Inhibition of HIV-1 integrase catalytic activity.
Table S3. Sequences of oligonucleotides and primers used to clone the peptides.

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