Interaction between HIV-1 Rev and Integrase Proteins

A BASIS FOR THE DEVELOPMENT OF ANTI-HIV PEPTIDES

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Joseph Rosenbluh,1 Zvi Hayouka,1 Shoshana Loya,1 Avid Levin,1,1, Ayelet Armon-Omer,1, Elena Britan,1, Amnon Hizi,1 Moshe Kotler,1 Assaf Friedler,1,1, and Abraham Loyter1,2

From the 1Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, and 2Department of Organic Chemistry, Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, the 3Department of Cell and Developmental Biology, The Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, and the 4Department of Pathology, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Human immunodeficiency virus 1 (HIV-1) Rev and integrase (IN) proteins are required within the nuclei of infected cells in the late and early phases of the viral replication cycle, respectively. Here we show using various biochemical methods, that these two proteins interact with each other in vitro and in vivo. Peptide mapping and fluorescence anisotropy showed that IN binds residues 1–30 and 49–74 of Rev. Following this observation, we identified two short Rev-derived peptides that inhibit the 3′-end processing and strand-transfer enzymatic activities of IN in vitro. The peptides bound IN in vitro, penetrated into cultured cells, and significantly inhibited HIV-1 in multinuclear activation of a galactosidase indicator (MAGI) and lymphoid cultured cells. Real time PCR analysis revealed that the inhibition of HIV-1 multiplication is due to inhibition of the catalytic activity of the viral IN. The present work describes novel anti-HIV-1 lead peptides that inhibit viral replication in cultured cells by blocking DNA integration in vivo.

Following penetration of human immunodeficiency virus 1 (HIV-1)1,2 into the host cell, reverse transcription of the viral RNA produces cDNA that is then integrated into the host chromosome (1). The integration of the viral cDNA is an essential early step in the HIV-1 life cycle. This reaction is catalyzed by the viral integrase (IN), a 32-kDa protein that is an integral part of the viral pre-integration complex (PIC) (2). The IN protein is encoded by the viral pol gene and is translated as part of a large Gag-Pol polyprotein that is processed by the viral protease (3, 4).

For the integration process to occur, the viral IN must recognize specific sequences in the viral cDNA, at the termini of the long terminal repeat (LTR) elements. Retroviral integration proceeds in two steps: in the first, termed 3′-end processing, a dinucleotide is removed from the 3′-end. This reaction occurs in the cytoplasm, within the PIC (5, 6). In the next step, after entering the nucleus, the processed viral double-stranded DNA is joined to the host target DNA by an IN-mediated strand-transfer reaction.

Due to its central role in HIV replication, the IN protein is an attractive target for antiviral therapy (4). Identifying specific IN inhibitors may provide a novel approach for multi-therapy strategies. Moreover, probably no cellular counterpart of IN exists in human cells and therefore, IN inhibitors should not interfere with normal cellular processes. However, only a few IN inhibitors have been described to date (4). This is in contrast to the number of inhibitors obtained against reverse transcriptase (RT) and the protease, which are the other HIV-1 enzymes that are currently used as targets for the development of anti-HIV-1 drugs (4). A promising approach for the discovery of IN inhibitors is the use of peptides derived from the IN-binding sequences of IN-interacting proteins.

Specific domains within viral proteins are responsible for their interaction with host-cell receptors and with other viral and cellular proteins enabling the completion of the viral propagation cycle within the host cell (1, 6). Peptides derived from these binding domains may interfere with virus-host and virus-virus protein interactions and as such are excellent candidates as therapeutic agents. Using this approach, short peptides that inhibit IN enzymatic activity were obtained following analysis of the interaction between two of the HIV-1 proteins, RT and IN. Screening a complete library of RT-derived peptides demonstrated that two domains of about 20 amino acids mediate this interaction. Peptides bearing these amino acid sequences blocked IN enzymatic activities in vitro (7).

In the present work, we observed a not yet described interaction between the HIV-1 Rev and IN proteins. The HIV-1 Rev is a karyophilic protein, which is required at the late phase of the viral life cycle for promoting nuclear export of partially spliced or un-spliced viral RNA (8, 9). Based on this finding, we identi-
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fied two domains within the HIV-1 Rev protein that mediate its binding to IN. Peptides derived from these binding domains blocked IN enzymatic activity in vitro, penetrated into cultured cells, and inhibited viral cDNA integration and consequently HIV-1 replication.

EXPERIMENTAL PROCEDURES

Cells—Monolayer adherent HeLa and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium. The T-lymphocyte cell lines Sup T1 and H9, provided by the National Institutes of Health Reagent Program (Division of AIDS, NIAID, NIH), was grown in RPMI 1640 medium. HeLa MAGI cells (TZM-bi) (10), which express the β-galactosidase gene under regulation of the trans-activation response element (11), were obtained through the NIH Reagent Program, and grown in Dulbecco’s modified Eagle’s medium. Cells were incubated at 37 °C in 5% CO2 atmosphere. All media were supplemented with 10% (v/v) fetal calf serum, 0.3 g/liter l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Biological Industries, Beit Haemek, Israel).

Viruses—Wild-type HIV-1 was generated by transfection (12) of HEK293T cells with pSVC21 plasmid containing the full-length HIV-1HXB2 viral DNA (13). Wild-type and Δenv/VSV-G viruses were harvested from HEK293T cells 48 and 72 h post-transfection with pSVC21 Δenv. The viruses were stored at −75 °C.

Infection of Cultured Cells—Cultured lymphocytes (1 × 106) were centrifuged for 5 min at 2000 × g. The supernatant was aspirated and the cells were resuspended in 0.2 to 0.5 ml of medium containing virus at a multiplicity of infection (m.o.i.) of 0.1 and 2. Following absorption for 1 h at 37 °C, the cells were washed to discard unbound virus and incubated for an additional 1 to 10 days.

HIV-1 Titration MAGI Assay—Titration of HIV-1 was carried out by the MAGI assay, as described by Kimpton and Emerman (11). Briefly, TZM-bi cells were transfected to 96-well plates at 10 × 103 cells per well. On the following day, the cells were infected with 50 μl of serially diluted HIV-1 Δenv/ VSV-G virus in the presence of 20 μg/ml DEAE-dextran (GE Healthcare). Following a 2-h incubation, 150 μl of Dulbecco’s modified Eagle’s medium was added. Two days post-infection, cultured cells were fixed with 1% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde in PBS. Following an intensive wash with PBS, cells were stained with a solution of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl2, and 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Ornait, Israel). Blue cells were counted under a light microscope at ×200 magnification.

P24 Assay—H9 lymphoid cells were incubated with the indicated peptides for 2 h and following infection with wild-type HIV-1 at a m.o.i. of 0.1 (as described above) the cells were incubated for 10 days and the P24 content was estimated. Determination of HIV-1 P24 antigen was performed by using the capture assay kit (SAIC, AIDS Vaccine Program, Frederick, MD), according to the manufacturer’s instructions.

Plasmid Construction—All of the plasmids used in this study were constructed using PCR cloning techniques with the high-fidelity enzyme Platinum Pfu DNA polymerase (Invitrogen). Clones were subjected to automated DNA sequencing. For the bimolecular fluorescence complementation (BiFC) experiments, the yeast multicity shuttle vectors pRS423 (with HIS3 as the selective marker) and pRS426 (with URA3 as the selective marker), both with the ADH1 promoter, were used as the cloning plasmids (a kind gift from Dr. D. Engelberg, Alexander Silberman Institute, The Hebrew University of Jerusalem). The DNA-coding region of the two yeast green fluorescent protein (GFP) fragments (14), namely the N terminus (GN) containing GFP amino acids 1–154, and the C terminus (GC) containing GFP amino acids 155–239, were cloned into pRS423 and pRS426, respectively. A linker consisting of (GGS)5 was used to separate the inserted genes. The final vectors were termed GN-linker (cloned into pRS423) and GC-linker (cloned into pRS426). The coding sequences of full-length HIV-1 IN, Rev, and Tat were amplified by PCR and inserted in-frame into the corresponding sites of the GN-linker in the C-terminal fragments of the GN, resulting in GN-IN, GN-Rev, and GN-Tat.

For the co-immunoprecipitation experiments, mammalian Rev expression vectors was constructed by PCR amplification of HIV-1 Rev and ligation into the pcDNA3.1 (Invitrogen) expression vector. The IN mammalian expression was a generous gift from Dr. Z. Debyster (15).

BiFC Assay to Study Protein-Protein Interactions—The plasmids described above were transformed into the yeast strain EGY48 (Clontech) and the cells were grown on yeast nitrogen base medium lacking histidine and uracil. After 48 h at 30 °C, the plates were transferred to 23 °C for 2 to 3 days and the appearance of fluorescence in yeast cells was visualized by confocal microscope (MRC 1024 confocal imaging system, Biorad) as previously described (16).

Study of in Vivo Protein-Protein Interactions Using the Co-immunoprecipitation Method—HEK293T cells were transfected with 5 μg of the Rev and IN mammalian expression vector genes using the calcium phosphate method (17). After 48 h, cells were harvested and washed three times with PBS and then lysed by the addition of PBS containing 1% (v/v) Nonidet P-40. A sample from the lysate obtained was subjected to a SDS-PAGE gel and immunoblotted with either a monoclonal anti-Rev antibody (αRev) (NIH AIDS Research & Reference Reagent Program, catalog number 7376) or an antiserum raised against IN amino acids 276–288 (NIH AIDS Research & Reference Reagent Program catalog number 758) or an anti-actin (α-Actin) antibody (Santa Cruz).

The remaining lysate was incubated for 1 h at 4 °C with either the αRev or αIN antibodies. Following a 3-h incubation with protein G-agarose beads (Santa Cruz) at 4 °C, the samples were washed three times with PBS containing 1% Nonidet P-40. An SDS buffer was added to the samples and after boiling and running on an SDS-PAGE gel the membranes were immunoblotted with either αIN or αRev antibodies.

ELISA-based Binding Assays—Protein-protein binding was estimated using an ELISA-based binding assay exactly as previously described (18).

Protein Expression and Purification—Expression and purification of histidine-tagged Rev-GFP and GST-IN conjugates were performed as previously described (19, 20). The GST-IN expression vector was a generous gift from Dr. A. Cereseto (20). The histidine-tagged IN expression vector was a generous gift
from Dr. A. Engelman and its expression and purification were performed essentially as described previously (21).

**GST Pulsedown—**GST-IN or GST (15 μg) were incubated for 30 min at room temperature with 10 μl of glutathione beads (Sigma) in 200 μl of buffer A (100 mM NaCl, 5% glycerol, 1 μM dithiothreitol, and 50 mM Tris-HCl, pH 7.5) containing 0.25% Nonidet P-40. After washing with buffer A, the beads were resuspended in 200 μl of buffer A supplemented with 0.25% Nonidet P-40, 0.1% (v/v) sodium deoxycholate, and 2 μg of histidine-tagged GFP or Rev-GFP, for 30 min at room temperature. Following three washes with buffer A, SDS buffer was added and the samples were boiled and analyzed by Western blotting using anti-His tag antibody (Santa Cruz).

**Cell Penetration Experiments—**Fluorescein-labeled peptides at a final concentration of 10 μM in PBS were incubated with HeLa cells for 2 h at 37 °C. After three washes in PBS, non-fixed cells were visualized by a confocal microscope (MRC 1024 confocal imaging system, Bio-Rad).

**Qualitative in Vitro Integration Assays—**Monitoring of the 3'-end processing and strand-transfer activities of the IN, as well as preparation of the recombinant HIV-1 IN and the gel-purified oligonucleotides used for these enzymatic assays, were performed exactly as previously described (7).

**Quantitative in Vitro Integration Assay—**Determination of the IN enzymatic activity enzymes in a quantitative way was performed using a previously described assay system (22, 23). In this assay a oligonucleotide substrate of which one oligo (5'-ACTGCTAGAGATTTTCCACACTGAAATGTC) was labeled with biotin on the 3'-end and the other oligo (5'-GACCCTTTAGTCAGTGTGGAAAATC-CCACACTGAGTAGCTGGGA-3') was labeled with digoxigenin at the 5'-end. The final reaction mixture contained 390 nM IN, 1 μM double strand oligonucleotide DNA, 20 mM Hepes (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 10% Me₂SO, 5% PEG-8000, 0.1 mg/ml BSA at 40 μl. When peptide inhibition was tested the IN was preincubated with the peptide for 10 min prior to addition of the DNA substrate. Following a 1-h incubation at 37 °C, 60 μl of a buffer containing 20 mM Tris-HCl (pH 8), 400 mM NaCl, 10 mM EDTA and salmon sperm DNA was added. This overall integrase reaction was followed by an immunosorbent assay on avidin-coated plates as described (22).

**Peptide Synthesis, Labeling, and Purification—**Peptides were synthesized on an Applied Biosystems (ABI) 433A peptide synthesizer using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The amino acids were purchased from NOVAbiochem or Bio-Lab (Jerusalem, Israel). The peptides were labeled at their N terminus with 5'- and 6'-carboxyfluorescein succinimidyl ester (Molecular Probes) using a 4-fold excess of the fluorescein and 4-fold excess of hydroxybenzotriazole. The peptides were purified on a Gilson HPLC using a reverse-phase C8 semi-preparative column (ACE, C-8 RP) with a gradient from 5 to 60% buffer B in buffer A (buffer A, 0.001% (v/v) trifluoroacetic acid in water, and buffer B, 0.001% (v/v) trifluoroacetic acid in acetonitrile). The identity of the peptides was confirmed using an ABI Voyager matrix-assisted laser desorption ionization time-of-flight mass spectrometer. The sequences of the peptides are summarized in Table 1.

**Fluorescence Anisotropy—**Binding studies were performed at 10 °C using a PerkinElmer LS-50b luminescence spectrofluorometer equipped with a Hamilton microcarol M dispenser. The fluorescence-labeled peptides were dissolved in 20 mM Tris buffer (pH 7.4) at the desired ionic strength to a final concentration of 0.05 to 0.1 μM. A 1-ml aliquot of the peptide solution was placed in a cuvette, and 200 μl of 100 μM IN protein (IN molarity was calculated assuming IN monomer) was titrated into the peptide solution in 20 steps of 10 μl each at 15-min intervals. The total fluorescence and anisotropy were measured after each addition. The excitation wavelength was 480 nm and the emission wavelength was 530 nm. The bandwidths were changed depending on the concentration of the labeled molecule used. Data were fitted to the Hill equation, where \( R = R_0 + \frac{\Delta R \cdot (K_a \cdot [IN]^n)}{1 + K_a \cdot [IN]^n} \) (Eq. 1)

where \( R \) is the measured fluorescence anisotropy value, \( \Delta R \) is the amplitude of the fluorescence change from the initial value (peptide only) to the final value (peptide in complex), \([IN]\) is the protein concentration added, \( R_0 \) is the starting fluorescence anisotropy value, corresponding to the free peptide, and \( K_a \) is the association constant, which is equal to \( 1/K_d \) (the dissociation constant).

**Quantization of Integrated HIV-1 DNA in the Cellular Genome—**Following incubation of Sup T1 cells with the indicated peptides for 2 h, the cells were infected with a HIV-1 Δenv/VSV-G virus at a m.o.i. of 2 (as described above) for 24 h. During the first round PCR, integrated HIV-1 sequences were amplified with the HIV-1 LTR-specific primer (LTR-TAG-F, 5'-ATGCCACGTAAACGAAAATCTTGCTAATAGGAAACCACCTG-3') and Alu-targeting primers (first-Alu-F, 5'-AGCTCCTCCGATGAGCTGGA-3' and first Alu-R, 5'-TTAAGCCGATGACCCCG-3') (24) that annealed to conserved regions of the Alu repeat element. Alu-LTR fragments were amplified from 1/10 of the total cell DNA in a 25-μl reaction mixture containing 1× PCR buffer, 3.5 mM MgCl₂, 200 μM dNTPs, 300 nM primers, and 0.025 units/μl of Taq polymerase. The first round PCR cycle conditions were as follows: a DNA denaturation and polymerase activation step of 10 min at 95 °C and then 12 cycles of amplification (95 °C for 15 s, 60 °C for 30 s, 72 °C for 2 min).

During the second round PCR, the first round PCR product could be specifically amplified by using the tag-specific primer (tag-F, 5'-ATGCCACGTAAACGAAAATCTG-3') and the LTR primer (LTR-R, 5'-AGCCAAAGCTTTATGCGTAAAG-3') designed by Primer Express (Applied Biosystems) using default settings. The second round PCR was performed on 1/25 of the first round PCR product in a mixture containing 300 nM of each primer, 12.5 μl of 2× SYBR Green master mixture (Applied Biosystems) at a final volume of 25 μl, run on an ABI PRISM 7700 (Applied Biosystems). The second round PCR cycles began with DNA denaturation and a polymerase-activation step (95 °C for 10 min), followed by 50 cycles of amplification (95 °C for 15 s, 60 °C for 60 s). For generating a linear curve the SVC21 plasmid containing the full-length HIV-1Env viral DNA was used as a template. In the first round PCR the LTR-
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TAG-F and LTR-R primers were used and the second round PCR was performed using the tag-F and LTR-R primers. The standard linear curve was in the range of 5 ng to 0.25 fg (r = 0.99). DNA samples were assayed with quadruplets of each sample.

PCR Analysis of Early Viral Genes—Sup T1 cells were incubated with 12.5 μM of either peptides or with 2 μM azidothymidine for 2 h, cells following infection with a HIV-1 Δenv/VSV-G virus at a m.o.i of 2, and incubation for 6 h. The viral Gag or Nef DNA sequences were amplified using specific primers: Gag-specific primers, 5′-AGTGCCCCAGAGCTGAAAG-3′ and 5′-TGCTAGTCGGAGCCATG-3′ and Nef-specific primers, 5′-CCTGGCTAGAAGCACAAGAG-3′ and 5′-CTTGTAGCAAGCCTGATGC-3′. The fragments were amplified from 10 ng of the total cell DNA in a 25-μl reaction mixture containing 1× PCR buffer, 3.5 mM MgCl₂, 200 μM dNTPs, 300 nM primers, and 0.025 units/μl of Taq polymerase. The PCR conditions were as follows: a DNA denaturation and polymerase activation step of 5 min at 95 °C and then 29 cycles of amplification (95 °C for 45 s, 60 °C for 30 s, 72 °C for 45 s).

Effect of Peptides on Cell Viability Using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) Assay—Following incubation with the indicated peptides, the medium was removed and the cells were incubated in Earle’s solution containing 0.3 mg/ml MTT for 1 h. Subsequently, the solution was removed and the cells were dissolved in 100 μl of Me₂SO for 10 min at room temperature. The Me₂SO-solubilized cells were transferred to a 96-well ELISA plate, and OD values were monitored at a wavelength of 570 nm (25).

RESULTS

HIV-1 IN and Rev Proteins Interact When Expressed in Yeast and Mammalian Cultured Cells—Using the BiFC assay, we show that the HIV-1 Rev and IN proteins interact with each other in yeast cells (Fig. 1). In this assay, two proteins of interest are fused to the non-fluorescent N- or C-terminal halves of the GFP molecule (termed GN and GC). Intracellular restoration of the fluorescence of GFP indicates interaction between the two fused proteins (14, 26). An interaction between the Rev and IN proteins can be inferred from the appearance of fluorescence within yeast cells transfected with plasmids bearing the coding sequence of these two proteins fused to GFP fragments (Fig. 1, a and b). The fluorescence is seen within the cells’ nuclei, suggesting that the Rev-IN interaction either does not disturb the karyophilic properties of these two proteins (27, 28) or occurs following their nuclear import (see Fig. 1f).

In vitro binding assay systems, with recombinant purified proteins, were used to further characterize the interaction between Rev and IN. Because the recombinant Rev protein is highly insoluble (31), we used a Rev-GFP conjugate that is soluble and func-
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Inhibition of IN Catalytic Activity by Rev-derived Peptides—A qualitative assay, which monitors both the strand-transfer and 3' processing, was used to study the inhibitory effect of the Rev-derived peptides. In the strand-transfer assay, the integration of the labeled 5'-end substrate that serves as both the target and donor DNA resulting in an increase of the molecular size of the substrate. In the 3'-end processing assay, the unique cleavage of the labeled substrates is followed (33). No significant inhibition of IN activity by the full-length Rev protein was observed (not shown), nor was such an effect observed by Rev49–74 (Fig. 5e, in which results of the strand-transfer assay are shown). Similar results, namely no inhibitory effect, were obtained when the 3'-end processing activity was assayed (not shown). However, a low degree of inhibition was observed by Rev1–30 at a concentration of 300 μM (Fig. 5d). As already mentioned, peptides derived from the IN-binding regions of IN-binding proteins, such as RT, can potentially inhibit IN activity (7). The interaction between Rev and IN, discovered here, led us to explore the possibility that Rev-derived short peptides may affect IN activity in a similar manner. Consequently, a peptide library spanning the full-length HIV-1 Rev subtype B consensus sequence was screened for inhibition of IN enzymatic activities. This library, obtained from the NIH AIDS Research & Reference Reagent Program, contains small samples of 27 peptides, each 15 amino acids in length, with an 11-amino acid overlap between sequential peptides (Table 2). Six peptides corresponding to two regions of the Rev protein inhibited IN 3'-end processing activity (Fig. 6a and Table 2). Four of these six inhibitory peptides blocked IN strand-transfer activity (Fig. 6b and Table 2).

Binding to IN and Penetration into Cultured Cells of the Rev-derived Inhibitory Short Peptides—Of the six Rev-derived inhibitory peptides, the sequences of peptides Rev9–23 (5993) and Rev53–67 (6004) (Table 2) were selected for further study, including binding to IN, penetration into cultured cells, and inhibition of HIV-1 replication. For these studies, we synthesized two peptides: 1) one corresponding to 5993 but lacking its first four amino acids (DEEL) (Rev13–23, Table 1), to obtain a cell-permeable short peptide deficient of the negatively charged amino acids, and 2) one bearing the complete sequence of peptide 6004 (Rev53–67, Table 1). Similar to the corresponding peptides from the NIH peptide library (peptides 5993 and 6004), peptides Rev13–23 and Rev53–67 inhibited strand transfer (Fig. 5f and g) and 3'-end processing (not shown). The inhibitory effect of the Rev13–23 and Rev53–67 peptides was confirmed by using a quantitative integration assay system (Fig. 6c). This assay allowed to demonstrate that IN inhibition is peptide concentration dependent with an apparent IC₅₀ of 25 μM for both peptides (Fig. 6c). Fluorescence anisotropy studies (Fig. 4b) revealed that peptides Rev13–23 and Rev53–67 inter-

Table 1) covering the full-length sequence of the Rev protein were synthesized and their interaction with IN was studied using fluorescence anisotropy. Rev1–30 and Rev49–74 bound to an IN with Kᵣ values at the low micromolar range, and a Hill coefficient of around 4, indicating binding of IN tetramer to the peptides (Fig. 4a and Table 1). Peptides Rev31–48, Rev74–93, and Rev94–116 failed to show any binding to IN (Fig. 4a and Table 1).

Inhibition of IN Catalytic Activity by Rev-derived Peptides—A qualitative assay, which monitors both the strand-transfer and 3' processing, was used to study the inhibitory effect of the Rev-derived peptides. In the strand-transfer assay, the integration of the labeled 5'-end substrate that serves as both the target and donor DNA resulting in an increase of the molecular size of the substrate. In the 3'-end processing assay, the unique cleavage of the labeled substrates is followed (33). No significant inhibition of IN activity by the full-length Rev protein was observed (not shown), nor was such an effect observed by Rev49–74 (Fig. 5e, in which results of the strand-transfer assay are shown). Similar results, namely no inhibitory effect, were obtained when the 3'-end processing activity was assayed (not shown). However, a low degree of inhibition was observed by Rev1–30 at a concentration of 300 μM (Fig. 5d). As already mentioned, peptides derived from the IN-binding regions of IN-binding proteins, such as RT, can potentially inhibit IN activity (7). The interaction between Rev and IN, discovered here, led us to explore the possibility that Rev-derived short peptides may affect IN activity in a similar manner. Consequently, a peptide library spanning the full-length HIV-1 Rev subtype B consensus sequence was screened for inhibition of IN enzymatic activities. This library, obtained from the NIH AIDS Research & Reference Reagent Program, contains small samples of 27 peptides, each 15 amino acids in length, with an 11-amino acid overlap between sequential peptides (Table 2). Six peptides corresponding to two regions of the Rev protein inhibited IN 3'-end processing activity (Fig. 6a and Table 2). Four of these six inhibitory peptides blocked IN strand-transfer activity (Fig. 6b and Table 2).

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act with IN, showing binding affinities similar to those obtained with the longer Rev peptides (see Fig. 4b and Table 1). The specificity of peptide binding is demonstrated by the results showing that scrambled Rev peptides that contain the sequence of Rev13–23 and Rev53–67 in a random order, failed to interact with the IN protein (Fig. 4b and Table 1). Also, no inhibition of the IN enzymatic activity by the scrambled Rev-derived peptides was observed using either the quantitative (Fig. 6c) or by the qualitative (not shown) integration assay systems.

Incubation of the fluorescnetly labeled Rev13–23, Rev53–67, or scrambled Rev13–23 with HeLa cultured cells resulted in the appearance of intracellular fluorescence, as was observed by Western blotting using a monoclonal anti-His antibody.

**TABLE 1**

| Peptide name       | Amino acid sequence | $K_d$ binding to IN | Hill coefficient |
|--------------------|---------------------|---------------------|------------------|
| Rev1–30            | MAGRSGDSDEELLKTVRLIKFLYQSNPPPS | $6.5 \pm 0.2$       | 4.4              |
| Rev31–48           | FEGTRQARRRRRGRWRER   | $11.2 \pm 0.5$      | 4.9              |
| Rev49–74           | QGRIGRSGVNLSTYGLGPRAEFPVLQ | $11.2 \pm 0.5$      | 4.9              |
| Rev74–93           | QPLPRLRTILCNEDCOTSG  | TLTM                | 4.9              |
| Rev94–116          | TQGVSQPILVESPAVELSOTE | TLTM                | 4.9              |
| Rev13–23           | LKTVRFLKFLY          | $2.8 \pm 0.1$       | 3.6              |
| Rev53–67           | RSIGNILSTYGLGPR      | $6.9 \pm 0.1$       | 5.2              |
| IN5                | FVSTHSVPSWPWLIDLIV    | $7.5 \pm 0.2$       | 6.0              |
| Rev13–23 scrambled | FRKLLYLTKVL          | TLTM                | 6.0              |
| Rev53–67 scrambled | LGYRTFSGRWSI         | TLTM                | 6.0              |

* TLTM, too low to measure.

*Fig. 3. In vitro binding of Rev and IN.* Plates coated with Rev-GFP (a and b) were blocked with 5% BSA. Following washing, biotinylated BSA-IN or biotinylated BSA (Bb) at the indicated concentrations (a) or 10 nM biotinylated BSA-IN preincubated with various molar ratios of Rev-GFP or carbonic anhydrase (b) were added. All other experimental conditions, including the estimation of bound biotin molecules, were as described under “Experimental Procedures.” c, purified GST-IN or GST were incubated with histidine-tagged Rev-GFP or GFP and after precipitation with glutathione beads and washing, were analyzed by Western blotting using a monoclonal anti-His antibody.
confocal microscopy, indicating their translocation via the cells’ plasma membrane (Fig. 7, a–c). On the other hand, the scrambled Rev53–67 and the Rev1–30 peptides were unable to penetrate the same cells (Fig. 7, d and e).

The Rev-derived Short Peptides (Rev13–23 and Rev53–67) Inhibit HIV-1 Replication in Cultured Cells at Non-toxic Concentrations—The effect of the Rev-derived Rev13–23 and Rev53–67 peptides on HIV-1 propagation was studied using TZM-bl (MAGI) cells (10). The percentage of blue cells following infection with HIV-1 indicates the titer of the infectious virus (11). Fig. 8a shows that both peptides inhibit HIV-1 replication in a concentration-dependent manner. Almost complete inhibition of HIV-1 replication was observed with peptide Rev13–23 at a concentration as low as 2.5 μM, whereas about 70% inhibition was observed with the same concentration of peptide Rev53–67. No synergetic effect was observed when a mixture of the two peptides was added (Fig. 8a), suggesting that both peptides interact with the same IN domain. A sharp decrease in the amount of viral P24 was also obtained in infected lymphoid cells incubated with peptides Rev13–23 and Rev53–67 (Fig. 8b). The degree of inhibition observed with both peptides was very close to that obtained with the classical anti-HIV-1 RT inhibitor azidothymidine (Fig. 8b). No inhibition of HIV-1 replication in MAGI or lymphoid cells was observed when the scrambled Rev peptides were used, indicating that HIV-1 inhibition by the Rev derived peptides is sequence specific (Fig. 8, a and b).

The Rev-derived peptides blocked HIV-1 replication by inhibiting the viral DNA integration step, as estimated by real time PCR in infected lymphoid cells (Fig. 8c). The results in Fig. 9, a and b, demonstrate that at the concentrations that the Rev-derived peptides inhibited viral DNA integration (Fig. 8c) no inhibition was observed in the amount of total viral DNA (Fig. 9). These results indicate that the peptides did not inhibit early virus infection steps such as cell penetration or reverse transcription. At the experimental conditions described the PCR was at the linear curve as is clearly indicated by results showing that the amount of the amplified DNA is increased with increasing PCR cycle numbers (supplemental Fig. S3). At the concentrations used, the Rev-derived peptides were not only non-toxic but, as reflected by the MTT assay, increased cell viability by about 50% (Fig. 8d). Because the MTT assay is based on the activity of a mitochondrial reductase (25) it is possible that the Rev peptides cause un-specific stimulation of this enzyme.

We also selected IN-binding peptides using the yeast two-hybrid screening system with a random peptide library (34, 35).
Indeed, an IN-binding peptide was selected and designated IN5 (see supplemental data and Table 1). IN5 interacts with the IN protein as was determined by fluorescence anisotropy (see supplementary Fig. S1a). However, IN5 was practically unable to block IN enzymatic activity (Fig. 5c) or HIV-1 replication (Fig. 8, a–c), despite its ability to penetrate cultured cells (see supplemental Fig. S1b). These results support the view that a peptide, which does not inhibit IN activity in vitro, cannot block HIV replication in vivo.

**DISCUSSION**

The interaction between Rev and IN, described for the first time in the present work, was observed in vitro as well as in the intracellular environment of yeast and mammalian cultured cells. This interaction did not have any effect on the biological functions of the IN protein as was inferred from experiments showing that HIV-1 replication was the same in cells lacking or overexpressing Rev protein (supplemental Fig. S2). The notion that the Rev protein does not affect IN enzymatic activities was further confirmed by in vitro experiments (not shown). Also, Rev49–74 peptide, bearing the Rev domain that is putatively involved in IN binding, did not inhibit IN enzymatic activity, but on the other hand Rev1–30 did cause slight inhibition. Interestingly, short 15-mer Rev-derived IN-binding peptides inhibited IN activity. It is possible that the observed inhibition of IN is due to steric hindrance of its active site that is accessible.
only to the short Rev-derived peptides but not to the full-length Rev protein.

The two Rev-derived peptides, Rev13–23 and Rev53–67, blocked HIV-1 replication in cultured cells at low micromolar concentrations, as estimated by three different unrelated assay systems. A clear correlation exists between the ability of these two peptides to inhibit IN enzymatic activities in vitro and their ability to reduce HIV-1 replication, indicating inhibition of the viral IN in vivo. This is particularly well exemplified by the experiments showing that the IN-binding, cell-permeable IN5 peptide, selected using the yeast two-hybrid system, does not block HIV-1 replication. This peptide differs from the inhibitory Rev13–23 and Rev53–67 peptides only in its inability to block IN activity in vitro. The view that the interaction between the Rev-derived peptides and IN is sequence specific and not mediated by nonspecific hydrophobic interactions is further strengthened by the results showing that the two scrambled peptides bearing the same amino acids as the inhibitory Rev-derived peptides did not have any binding or inhibitory activities.

Studies of the integration process in vivo by real time PCR further supported the view that the reduction in HIV-1 replication observed in the presence of the peptides Rev13–23 and Rev53–67 is due to inhibition of IN catalytic activity. This conclusion is further confirmed by the experiments demonstrating that no reduction in the total viral DNA was observed in the presence of the inhibitory peptides in HIV-1-infected cells. Furthermore, preliminary in vitro experiments have clearly demonstrated that the Rev13–23 and Rev53–67 peptides do not inhibit RT enzymatic activity (not shown).
A limited number of IN inhibitory peptides have already been described. Using a combinatorial peptide library, Plasterk and co-workers (36) selected a hexapeptide bearing the sequence HCKFWW that inhibited the 3′-processing and integration activity of IN. Based on the observation that this peptide also inhibited the IN from HIV-2, FIV, and MLV, it was suggested that a conserved region around the catalytic domain of IN is being targeted. An IN inhibitory peptide was also selected using a phage display library (37). IN-derived peptides that interfered with its oligomerization also blocked its enzymatic activity (38). Several other inhibitory peptides have been described in the last few years (39), but no attempts have been made to find out whether any of the described inhibitory peptides also block enzyme activity in vivo or, alternatively, HIV replication. To the best of our knowledge, only one study describes an IN inhibitory peptide that, similar to the peptides identified in the present work, also blocked HIV-1 replication in MAGI cells. However, efficient inhibition (75–85%) was obtained only at high peptide concentrations, in the order of 100 μM (34).

Because IN is an integral part of the PIC, it is unlikely that it will encounter the Rev protein that is a post-transcriptional regulator (40). Thus, the biological significance of our finding regarding the interaction between IN and Rev is, as yet, unclear. However, our studies reveal novel compounds with therapeutic potential against HIV-1. Attempts to elucidate the relevance of the Rev-IN interaction to the viral replication cycle are currently under investigation in our laboratories.

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