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Marlène Maroun, Olivier Delelis, Gaël Coadou, Thomas Bader, Emmanuel Ségéral, et al.. Inhibition of Early Steps of HIV-1 Replication by SNF5/Ini1. *Journal of Biological Chemistry*, 2006, 281 (32), pp.22736-22743. 10.1074/jbc.M604849200 . hal-03140758

HAL Id: hal-03140758

<https://hal.science/hal-03140758v1>

Submitted on 13 Feb 2021

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Inhibition of Early Steps of HIV-1 Replication by SNF5/Ini1^{*S}

Received for publication, May 19, 2006 Published, JBC Papers in Press, June 13, 2006, DOI 10.1074/jbc.M604849200

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To replicate, human immunodeficiency virus, type 1 (HIV-1) needs to integrate a cDNA copy of its RNA genome into a chromosome of the host cell, a step controlled by the viral integrase (IN) protein. Viral integration involves the participation of several cellular proteins. SNF5/Ini1, a subunit of the SWI/SNF chromatin remodeling complex, was the first cofactor identified to interact with IN. We report here that SNF5/Ini1 interferes with early steps of HIV-1 replication. Inhibition of SNF5/Ini1 expression by RNA interference increases HIV-1 replication. Using quantitative PCR, we show that both the 2-long terminal repeat circle and integrated DNA forms accumulate upon SNF5/Ini1 knock down. By yeast two-hybrid assay, we screened a library of HIV-1 IN random mutants obtained by PCR random mutagenesis using SNF5/Ini1 as prey. Two different mutants of interaction, IN E69G and IN K71R, were impaired for SNF5/Ini1 interaction. The E69G substitution completely abolished integrase catalytic activity, leading to a replication-defective virus. On the contrary, IN K71R retained *in vitro* integrase activity. K71R substitution stimulates viral replication and results in higher infectious titers. Taken together, these results suggest that, by interacting with IN, SNF5/Ini1 interferes with early steps of HIV-1 infection.

The replication cycle of human immunodeficiency virus, type 1 (HIV-1)⁴ involves the insertion of a DNA copy of its RNA

genome into a chromosome of the host cell. Following retrovirus entry, a large nucleoprotein complex called preintegration complex (PIC) is formed in the cytoplasm with components of the virion core and cellular factors. In addition to viral cDNA, PICs contain several viral proteins: matrix, nucleocapsid, reverse transcriptase, VPR protein, and integrase (IN), which carries out DNA-cutting and -joining reactions (1, 2). HIV-1 IN consists of three functional domains: the N-terminal domain (residues 1–49), the catalytic core domain (residues 50–212), and the C-terminal domain (residues 213–288) (3, 4). The N-terminal domain contains an HHCC motif that binds one Zn²⁺ atom and is involved in the multimerization of the protein (4). The C-terminal domain binds DNA non-specifically and plays a role in the formation of an active multimer of IN (3). The catalytic core domain contains the canonical 3-amino acid motif, D, D(35)E, that is essential for the catalytic activity of the protein (4). These residues coordinate a divalent metal ion (Mg²⁺) and are highly conserved among all integrases and retrotransposases. Integration proceeds in three steps, 3'-processing, strand transfer, and gap repair. Purified IN exhibits both 3'-processing and strand transfer *in vitro*. Double-stranded blunt-ended viral DNA produced by reverse transcription is first cleaved immediately 3' of a conserved CA dinucleotide motif. This reaction generates CA-3'-hydroxyl DNA ends that are the active intermediates of the strand transfer reaction. Both viral DNA ends are then inserted into a host cell chromosome. Finally, gap filling of the unrepaired 5'-ends of the viral DNA is under the control of cellular enzymes. In addition to the integration reaction, non-homologous end-joining and homologous recombination cellular pathways are involved in the formation of 1 and 2-LTR circles that are detected in the nuclei of infected cells (5–7).

Cellular factors, such as SNF5/Ini1 (8), LEDGF/p75 (9–14), EED (15), Rad18 (16), and HSP60 (17), were characterized to interact directly with IN. Other cellular proteins, such as HMG1a and Barrier of Autointegration Factor (BAF) interact with viral cDNA and participate in the integration reaction (18, 19). While *in vivo* HIV-1 integration is not sequence specific, transcriptionally inactive regions of the genome, such as centromeres and telomeres, are disfavored targets (20–22). Integration of proviral HIV-1 DNA occurs preferentially into transcriptional units of active genes, whereas the oncoretrovirus

fluorescence assay; GFP, green fluorescent protein; VSR-G, vesicular stomatitis virus-glycoprotein.

* This work was supported in part by grants from the Agence Nationale de Recherche sur le SIDA (ANRS), Sidaction, Association Pour la Recherche sur le Cancer, and by EC project "Hidden HIV Challenge" (FP6-2003-LIFESCI-HEALTH-3/012182) (to S. E.). The Benarous and Mouscadet laboratories are supported by EC project TRIoH (LSHB-CT-2003-503480). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

¹ Supported by doctoral fellowships from ANRS and Fondation pour la Recherche Medicale.

² Supported by a postdoctoral fellowship from EC project TRIoH (LSHB-CT-2003-503480).

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⁴ The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; PIC, preintegration complex; IN, integrase; LTR, long terminal repeat; siRNA, short interference RNA; m.o.i., multiplicity of infection; GST, glutathione S-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; WT, wild type; HTRF, homogenous time-resolved

murine leukemia virus shows integration preference near the transcription start sites of actively transcribed genes (23, 24). The differences observed between the integration profiles of these two viruses strongly suggest that cellular cofactors actively tether proviral DNA to specific regions of the genome (25).

SNF5/Ini1 was the first host protein identified as an IN-interacting factor by two-hybrid screenings (8). SNF5/Ini1 is one of the core subunits of the ATP-dependent chromatin remodeling complex SWI/SNF that regulates expression of numerous eukaryotic genes by altering DNA/histone interactions (26, 27). This complex was recently shown to be directly involved in Tat-mediated activation of HIV-1 transcription (28, 29). Moreover, SNF5/Ini1 was found to act as a tumor suppressor that is mutated in children with Malignant Rhabdoid Tumor (30). Further studies have shown that SNF5/Ini1 regulates cell proliferation by inhibiting activation of E2F-dependent genes through the p16ink4a-CDK4/Cyclin D-Rb pathway (reviewed in Ref. 31). Recently it was reported that, through the same pathway, SNF5/Ini1 controls chromosomal stability (32). SNF5/Ini1 was also found to interact with viral proteins such as Epstein-Barr virus nuclear protein 2 (EBNA2) (33) and human papillomavirus E1 (34) as well as cellular proteins ALL1 (35), c-Myc (36), and p53 (37).

The exact role of SNF5/Ini1 in HIV-1 replication remains unclear. Recombinant SNF5/Ini1 stimulates IN catalytic activity *in vitro* (8). When overexpressed, a cytoplasmic fragment of SNF5/Ini1 was able to interact with IN in the context of the Gag-pol precursor and in addition was reported to inhibit viral particle production, suggesting a role during the late stage of HIV-1 replication (38). Furthermore, SNF5/Ini1 was shown to be packaged in HIV-1, but not HIV-2 or simian immunodeficiency virus, viral particles (39). Interestingly, it has been observed that HIV-1 infection induces the cytoplasmic relocation of SNF5/Ini1 along with PML, leading to their association with incoming PIC before nuclear migration (40). However, the cytoplasmic accumulation of PML observed after retroviral infection is independent of the presence of SNF5/Ini1 (41). Furthermore, a direct effect of PML on HIV-1 infectivity was recently challenged (42). It has also been postulated that SNF5/Ini1 could target PICs to regions of the genome that are enriched for the SWI/SNF complex (43).

Using siRNA-mediated silencing of SNF5/Ini1 expression, we found that SNF5/Ini1 impairs early steps of HIV-1 replication by inhibiting formation of 2-LTR circle and integrated forms of viral DNA. We show that a single amino acid change, K71R, in integrase that reduces its ability to interact with SNF5/Ini1 leads to an increase in viral infectivity. Our results highlight the role of the interaction between SNF5/Ini1 and the incoming IN during early steps of the HIV-1 life cycle.

MATERIALS AND METHODS

Integrase Mutant Library—Yeast two-hybrid screening procedures were performed as previously described (14).

Plasmids—The GFP-IN^s expression vector was generated as previously described (14). Mutations were incorporated into the HIV-1_{Bru} molecular clones using PCR-directed mutagenesis as previously described (14). To generate the envelope-de-

leted NL4-3 vector (NL4-3 Δ env), a frameshift was introduced in the env gene. The NdeI site (nt6399) in pNL4-3 was digested, filled with Klenow, and religated. Wild-type and mutant integrases were inserted into bacterial expression vector pET15b (Novagene). GST-SNF5/Ini1 was constructed by PCR amplification from the SNF5/Ini1 expression vector (28) and subcloning into the pGEX4T1 expression plasmid (GE Healthcare).

Cells, Viruses, Transfections, and Infections—Human embryonic kidney 293, HeLa, and HeLa P4.2 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and antibiotics (100 units/ml penicillin G, 100 mg/ml streptomycin; Invitrogen). HeLa P4.2 cells were grown in the presence of 100 μ g/ml geneticin (Invitrogen). Jurkat and A3.01 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and antibiotics (100 units/ml penicillin G, 100 μ g/ml streptomycin; Invitrogen). A3.01 cells were transfected by nucleofection using the AMAXA cell line, Nucleofector Kit V, and program T014 following the manufacturer's instructions.

Virus stocks were produced by transfecting human embryonic kidney 293 cells using the calcium phosphate method with pBru-derived molecular clone. Single-round virus stocks were produced by co-transfecting pNL4-3 Δ env with vesicular stomatitis virus-glycoprotein (VSV-G) envelope expression vector. Supernatants were collected 2 days after transfection, and levels of HIV-1 p24 antigen were monitored by enzyme-linked immunosorbent assay (BD Biosciences). Jurkat cells were infected with viral doses corresponding to 30 ng of HIV-1 p24 antigen/10⁶ cells.

siRNA Knockdown Experiments—SNF5/Ini1 siRNAs (SNF5.1, GAGAUACCCUCACUCUGGTT, and SNF5.3, GAACUCAC-CAGAGAAGUUUTT) and control siRNAs (SNF5inv, GGUCU-CACUCCCAUAGAGTT, and GL2, CGUACGCGGAAUA-CUUCGATT) were synthesized by Eurogentec and annealed following the manufacturer's instructions. Jurkat T cells (2×10^6) were washed with PB-sucrose (phosphate-buffered sucrose buffer: 7 mM sodium phosphate, 272 mM sucrose, 1 mM MgCl₂) and then electroporated with siRNA at 1 mM with a Bio-Rad Gene Pulser II electroporator with an RF module (100 V, modulation 50%, 25 kHz, 10 bursts of 2 ms, burst interval 100 ms). HeLa or HeLa P4.2 cells were transfected twice at 24 h intervals with 10 or 30 nM siRNA using Oligofectamine reagent (Invitrogen).

Western Blot Analysis—Cells were lysed in radioimmune precipitation buffer containing 1 mM dithiothreitol and protease inhibitors. Proteins were separated by SDS-PAGE using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen) and revealed by Western blotting.

Antibodies—Rabbit polyclonal anti-SNF5 (raised against a synthetic peptide representing amino acids 370–386 of SNF5/Ini1 (HRNTRRRMRRLANTGPAAW)), mouse monoclonal anti-tubulin (clone DM 1A; Sigma). Secondary peroxidase-conjugated antibodies against mouse or rabbit immunoglobulins were purchased from Dako.

Quantification of Total HIV-1 DNA, 2-LTR Circles, and Integrated HIV-1 DNA—HeLa cells (2×10^5) were transfected with 30 nM siRNA. 24 h later, cells were washed three times with

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phosphate-buffered saline and infected with VSV-G-pseudotyped Bru virus (multiplicity of infection (m.o.i.) corresponding to 0.1). At different times postinfection cells were harvested, washed in phosphate-buffered saline, and treated for 1 h at 37 °C with 500 units of DNaseI (Roche Diagnostics) prior to DNA extraction using a QIAamp blood DNA mini kit (Qiagen). Quantifications were performed by real-time PCR on a LightCycler instrument (Roche Diagnostics). Sequences of primers and probes have been described previously (14). Copy numbers of total DNA, 2-LTR circle forms, and integrated DNA were determined in reference to standard curves prepared by amplification of cloned DNA with matching sequences (44). Results were normalized by the number of cells and the amount of cellular DNA quantified by PCR of the β -globin gene according to the manufacturer's instructions (Roche Diagnostics).

Expression and Purification of Recombinant Proteins and in Vitro Integration Assays—Recombinant GST-SNF5/Ini1 and N-terminal His-tagged IN were produced in *Escherichia coli* BL21. *E. coli* transformed with GST-SNF5/Ini1 expression plasmid were induced with 0.4 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h to induce protein expression. The bacterial pellet was resuspended in GST-lysis buffer A (20 mM Tris-HCl, pH 8.0, 500 mM KCl, 1 mM MgCl₂, 0.5% IGEPAL, 1 mM dithiothreitol, protease inhibitor mixture) (Sigma), and 1 mg/ml lysozyme was added. Cells were lysed by three cycles of freeze and thaw and then sonicated for 30 s. After centrifugation, the supernatant was incubated with glutathione-Sepharose for 1 h at 4 °C. The resin was washed four times with GST-lysis buffer and then twice with buffer B (20 mM Tris-HCl, pH 8.0, 20 mM KCl, 1 mM MgCl₂, 17% glycerol, 1 mM dithiothreitol). GST-SNF5/Ini1 was eluted from the resin in buffer B containing 25 mM reduced glutathione for 10 min at room temperature. His-IN mutants were purified by nickel affinity as previously described (45). Oligonucleotide substrates for IN reaction assays were as follows: U5B (5'-GTGTGGAA-AATCTCTAGCAGT-3'), U5B₋₂ (5'-GTGTGGAAAATCTCTAGCA-3'), and U5A (3'-CACACCTTTAGAGATCGTCA-5'). U5B or U5B₋₂ oligonucleotides were ³²P labeled using polynucleotide kinase and annealed to the complementary U5A oligonucleotide. IN activity reactions were carried out in a buffer containing 20 mM Hepes, pH 7.2, 1 mM dithiothreitol, and 10 mM MgCl₂. 3'-processing reactions were performed in the presence of 1.25 nM blunt IN substrate U5B/U5A. Strand transfer reactions were performed in the presence of 6.25 nM U5B₋₂/U5A substrate. ³²P-labeled duplex DNAs were incubated in 20 μ l of reaction buffer with 200, 400, or 600 nM integrase at 37 °C for 1 h. Reactions were stopped by adding 80 μ l of a stop solution (7 mM EDTA, 0.3 M sodium acetate, 10 mM Tris-HCl, pH 8). IN was extracted with phenol/chloroform. DNA fragments were ethanol precipitated, suspended in a loading dye, and separated on 18% polyacrylamide denaturing gels. Gels were analyzed on a STORM 840TM PhosphorImager (GE Healthcare).

Homogenous Time-resolved Fluorescence Assays (HTRF)—Assays were carried out in a black 384-halfwell microplate (Greiner) using the following assay buffer: 100 mM phosphate buffer, pH 7.0, 800 mM KF, 0.44 mM CHAPS, 10 μ M ZnCl₂, and 5

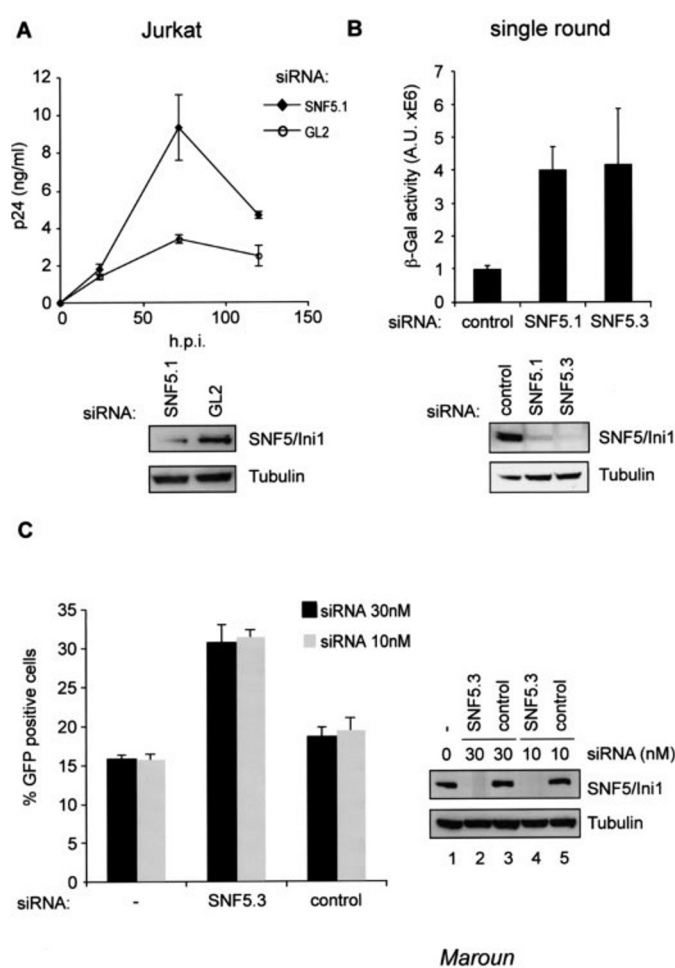


FIGURE 1. Inhibition of SNF5/Ini1 expression stimulates HIV-1 replication. *A*, Jurkat cells were transfected with 1 μ M SNF5/Ini1 (SNF5.1) or control (GL2) siRNAs. 24 h later, cells were infected with Bru virus. Viral replication was monitored by measuring p24 in cell supernatants. Errors bars represent standard deviation of three experiments. SNF5/Ini1 inhibition in these cells was detected 48 h after transfection by Western blotting of cell lysates using anti-SNF5/Ini1 antibody, or anti-tubulin antibody as a control. *B*, increase of HIV-1 replication after SNF5/Ini1 knock down in a single-round infection assay. HeLa P4.2 cells, expressing the β -galactosidase gene under the control of the HIV-1 LTR, were transfected twice with 30 nM SNF5/Ini1 (SNF5.1 and SNF5.3) or control (SNF5*inv*) siRNAs and then infected with NL4-3 Δ env virus pseudotyped with VSV-G virus. 24 h later, the β -galactosidase activity (arbitrary units) was monitored in cell lysates. This activity is the reflection of the amount of virus that infected the cells. The mean relative β -galactosidase activities (plus standard errors) obtained from three independent transfection experiments are shown. SNF5/Ini1 inhibition in these cells was detected 48 h after the second transfection by Western blotting of cell lysates using anti-SNF5/Ini1 antibody, or anti-tubulin antibody as a control. *C*, SNF5/Ini1 knock down stimulates HIV-GFP vector transduction. HeLa cells were transfected twice with two different amount of SNF5/Ini1 (SNF5.3), control (SNF5*inv*) siRNAs, or mock transfected. Cells were then infected with a GFP-expressing HIV vector and subjected to fluorescence-activated cell sorter analysis.

mM MgCl₂. Anti-GST cryptate (lot 49F) and anti-His-XL (lot 33F) from CisBio International were reconstituted as recommended. Protein concentrations and buffer conditions were previously optimized to result in an optimal signal. Consequently, recombinant His-tagged integrases were used at a final concentration of 50 μ g/ml, whereas GST-SNF5/Ini1 was at 0.25 μ g/ml. After addition of the interacting proteins and both antibodies on ice, the microplate was kept at 4 °C and read every 30 min in a Pherastar (BMG) at 665 and 620 nm after excitation

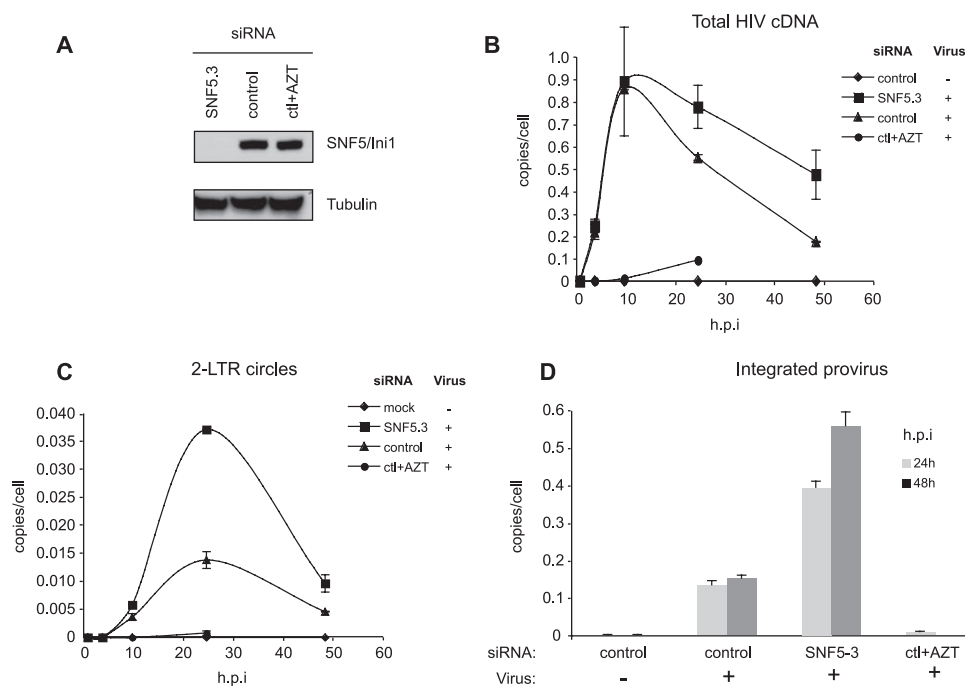


FIGURE 2. Stimulation of 2-LTR circular and integrated forms of HIV-1 upon SNF5/Ini1 knock down. A, HeLa cells were transfected twice with SNF5/Ini1 (*SNF5.3*) or control (*SNF5inv*) siRNAs and then infected with Bru virus pseudotyped with VSV-G envelope (m.o.i. corresponding to 0.1) or mock infected. At different time points following infection, cellular DNA was extracted and subjected to quantitative PCR analysis. Total viral DNA (B), 2-LTR circles (C), and integrated proviral DNA (D) were quantified. Error bars in panels B–D represent variations between duplicate quantitative PCR assays.

at 337 nm. All points were in quadruplicate. The specific HTRF signal was expressed as a percentage of ΔF and calculated as follows: $\Delta F (\%) = \{[(665/620) \text{ sample} - (665/620) \text{ blank}] / [(665/620) \text{ sample} + (665/620) \text{ blank}] \} \times 100$.

RESULTS

Transient Inhibition of SNF5/Ini1 Expression Stimulates HIV-1 Replication—To evaluate the importance of SNF5/Ini1 for viral replication, we first used RNA interference to knock down SNF5/Ini1 expression in different cell lines before infection with HIV-1 was performed. Jurkat cells were transiently transfected with siRNA directed against SNF5/Ini1 (*SNF5.1*) or control siRNA (*GL2*). Expression of SNF5/Ini1 was greatly reduced 48 h after treatment with *SNF5.1* siRNA but not with *GL2* siRNA, whereas the expression of tubulin was similar in both conditions (Fig. 1A, lower panel). When compared with cells treated with control siRNA, HIV-1 replication was stimulated up to 3-fold when SNF5/Ini1 expression was inhibited (Fig. 1A, upper panel). Because of the transient effect of siRNA, the increase in HIV-1 replication upon SNF5/Ini1 knock down was optimal at day 3. In a single-round assay using HeLa P4.2 reporter cells, silencing of SNF5/Ini1 gene expression using two different siRNA (*SNF5.1* or *SNF5.3*, Fig. 1B, lower panel) enhanced infection of NL4-3 Δenv virus pseudotyped with VSV-G by 3- to 4-fold (Fig. 1B, upper panel). These results indicate that inhibition of SNF5/Ini1 expression enhances early steps of HIV-1 replication. As expected, HeLa cells transfected with two different doses of *SNF5.3* siRNA also showed a 2-fold increase in transduction efficiency of an HIV-1 GFP reporter gene vector (Fig. 1C). Furthermore, the converse effect was

observed. When SNF5/Ini1 was transiently overexpressed in A3.01 cells, HIV-1 replication was reduced ~ 3 -fold 24 h postinfection. Overexpression of SNF5/Ini1 was confirmed by immunoblot analysis (Fig. 4C). All together, these data suggested that SNF5/Ini1 could negatively regulate an early step of HIV-1 replication.

Accumulation of 2-LTR Circles and Integrated HIV DNA Forms in SNF5/Ini1 Knockdown Cells—Because we observed that inhibition of SNF5/Ini1 expression led to an increase in HIV-1 replication, we next assessed which step in the virus life cycle is affected by SNF5/Ini1. We monitored the levels of total DNA, 2-LTR circles, and integrated forms of proviral HIV-1 DNA by quantitative PCR on cell extract from HeLa cells transfected with either siRNA control or siRNA against SNF5/Ini1 (*SNF5.3*). SNF5/Ini1 expression was efficiently inhibited 24 h after siRNA transient transfection (Fig. 2A). Cells were then infected with HIV-1 Bru pseudotyped with VSV-G envelope (m.o.i. 0.1) and harvested at 3, 9, 24, and 48 h postinfection. In HeLa cells, HIV-1 replication was restricted to a single round of infection. Levels of total HIV cDNA synthesis peaked at 9 h postinfection and were similar in cells treated with either control or *SNF5.3* siRNA. This amount remained higher at 24 and 48 h postinfection in cells where SNF5/Ini1 was inhibited (Fig. 2B). As expected, total HIV cDNA was barely detectable when infected cells were treated with a reverse transcriptase inhibitor, indicating that the quantitative PCR quantified *de novo* synthesized HIV cDNA. Interestingly, a 3- to 4-fold increase in 2-LTR circle forms was observed at 24 h postinfection in cells knocked down for SNF5/Ini1 (Fig. 2C). In addition, a 3- to 4-fold increase in the amount of integrated proviral DNA was also observed when SNF5/Ini1 expression was inhibited (Fig. 2D). Similar results were obtained when cells were transfected with a different siRNA (*SNF5.1*) and infected at a lower m.o.i. (data not shown). Thus, these data indicate that the increase in HIV-1 replication observed after inhibition of SNF5/Ini1 expression correlates with an increase in the number of integrated copies as well as 2-LTR circular forms.

Identification of Integrase Mutants Defective for SNF5/Ini1 Interaction—To further characterize the role of SNF5/Ini1 in HIV-1 replication, we used yeast two-hybrid screenings to select mutants of IN deficient for interaction with SNF5/Ini1. A library of random mutants of IN obtained by PCR was screened using SNF5/Ini1 as prey. Using a β -galactosidase assay, we selected and sequenced several IN mutants defective for SNF5/Ini1 binding. We focused on mutations affecting residues that

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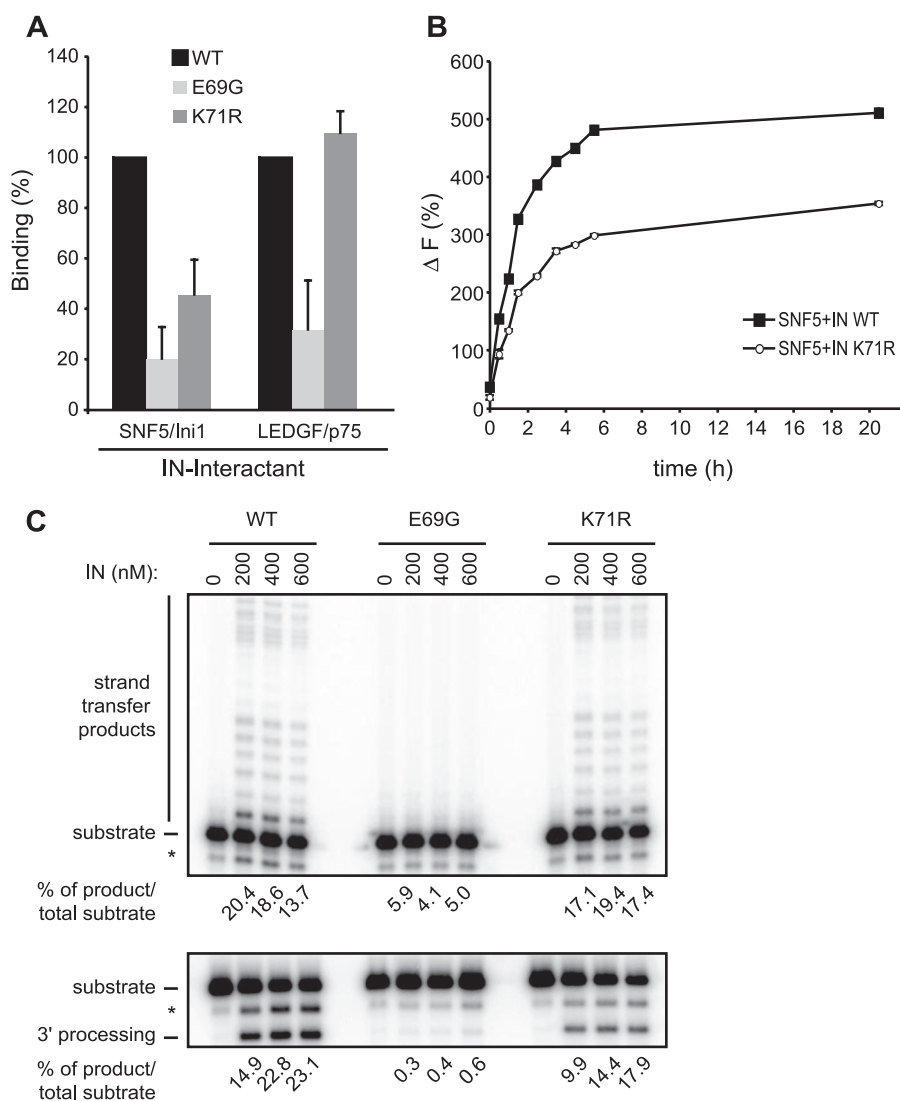


FIGURE 3. Characterization of IN mutants impaired in SNF5/Ini1 binding. A, interaction of wild type, E69G IN, or K71R IN with SNF5/Ini1 or LEDGF/p75 was tested using a two-hybrid quantitative β -galactosidase assay. B, time course detection of SNF5/Ini1-integrase complex by HTRF. GST-SNF5/Ini1 and His-IN WT or K71R were incubated with anti-GST-cryptate and anti-His-XL antibodies. HTRF signal was read at different time points in a Pherastar (BMG) at 665 and 620 nm after excitation at 337 nm. All points were in quadruplicate. The specific HTRF signal was expressed as a percentage of ΔF . C, activities of recombinant integrases were tested in an oligonucleotide-based assay. Both 3'-processing and strand transfer activities were evaluated for E69G and K71R IN in comparison with WT IN. Quantification of both activities is indicated as a percentage of product/substrate.

were exposed at the surface of the monomer, according to crystal structures (4). In particular, two specific mutants of IN, E69G and K71R, were of interest. Both residues were highly conserved among HIV-1 and SIVcpz strains (100% identity for Glu-69, 99.2% identity for Lys-71) (46). Interestingly, Glu-69 and Arg-166 are engaged in a hydrogen bond within the monomer. A virus harboring a K71E mutation was previously shown to be replication competent (47), whereas a double mutant, E69A/K71A, was described to be replication deficient (48).

To confirm that the loss of interaction of these IN mutants was specific, binding of IN wild type (IN WT), IN E69G, and IN K71R to either SNF5/Ini1 or LEDGF/p75 was quantified using a two-hybrid assay. IN E69G displayed only 20% binding to SNF5/Ini1 compared with the WT protein but was also impaired in its ability to bind to LEDGF/p75 (40% binding com-

pared with the WT). On the contrary, IN K71R was still able to bind LEDGF/p75, whereas it displayed 45% binding to SNF5/Ini1 (Fig. 3A). When the E69G and K71R mutations were introduced into an eukaryotic vector expressing GFP-IN fusion protein (49), subcellular localization analysis revealed that both WT and mutant GFP-IN accumulated in the nucleus (data not shown).

We developed an HTRF assay to quantify the interaction between SNF5/Ini1 and IN mutants (50). Time course experiments detecting formation of the IN-SNF5/Ini1 were performed. A rapidly increasing fluorescence resonance energy transfer signal was obtained between GST-SNF5/Ini1 and His-IN WT that was stabilized by 6 h and stable for up to 21 h. When His-IN K71R was used, an \sim 40% reduction of the signal was observed, confirming that this mutation partially reduced its ability to bind SNF5/Ini1 (Fig. 3B).

Finally, we analyzed the effects of these mutations on the catalytic activity of IN *in vitro*. Both 3'-processing and strand transfer activities were assayed in Mg^{2+} -containing buffer, using increasing amounts of recombinant proteins. IN K71R retained activities comparable with that of the WT protein. In contrast, IN E69G was impaired in both 3'-processing and strand transfer activities, likely to be because of the loss of Glu-69-Arg-166 interaction within the monomer (Fig. 3C). These data suggested that the overall structure of IN E69G was somehow disrupted, whereas IN K71R

was impaired in SNF5/Ini1 binding but retained a full catalytic activity *in vitro*.

Increase in Viral Infectivity of HIV-1 K71R Virus—To determine whether these residues were involved in viral replication, mutations were introduced into an HIV-1 molecular clone and viral stocks were produced in 293 cells. Viral particle production, as measured by HIV-1 p24 antigen release into the culture supernatant, was not affected in the clones harboring mutated integrases. To analyze the effect of these mutations on viral replication, we first measured the infectivity of the viral stocks. Different dilutions of viral stocks were used to infect HeLa P4.2 cells in a single-round assay allowing the quantification of the m.o.i. of the viruses. Each viral stock was then normalized for HIV-1 p24 viral antigen to quantify viral infectivity. As shown in Fig. 4A, substitution of integrase Lys-71 to Arg increased viral infectivity by 2-fold when

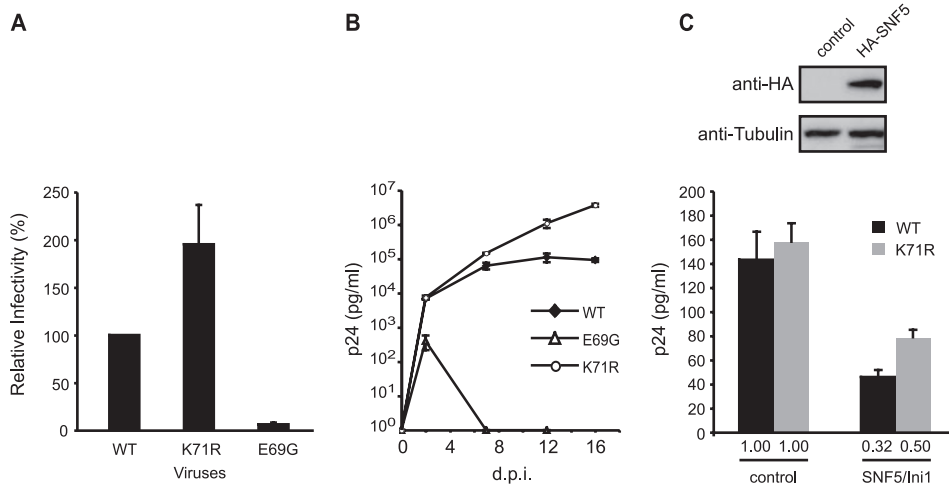


FIGURE 4. K71R mutation increases viral infectivity. A, infectivity of HIV-1_{WT}, HIV-1_{E69G}, and HIV-1_{K71R} was measured in a single-cycle assay on HeLa P4.2 cells and was normalized for the amount of p24 viral antigen of the virus stocks. Values are expressed as percentages of HIV-1_{WT} and represent the means and standard deviation of three independent experiments. B, Jurkat cells were infected with HIV-1_{WT}, HIV-1_{E69G}, and HIV-1_{K71R} (25 ng of p24 antigen/10⁶ cells), and viral accumulation was quantified by measuring p24 antigen in the supernatant of the cells. Values represent the means and standard deviation of three independent experiments. C, transient overexpression of SNF5/Ini1 impairs HIV-1 replication. A3.01 cells were transfected with control or HA-SNF5/Ini1 expression plasmids. Cells were infected with HIV-1_{WT} or HIV-1_{K71R} (m.o.i. 10⁻³), and 24 h postinfection viral accumulation was quantified by measuring p24 antigen in the supernatant of the cells. Errors bars represent standard deviation of three experiments. Ratio of p24 in HA-SNF5/Ini1 versus control cells is indicated for each virus. SNF5/Ini1 overexpression in these cells was detected 24 h after transfection by Western blotting of cell lysates using anti-HA antibody, or anti-tubulin antibody as a control.

Jurkat cells were infected using an amount of virus equivalent to 25 ng of p24 (corresponding to a m.o.i. of 4 × 10⁻⁴), and supernatants were collected every 2–3 days. As expected, HIV-1_{E69G} was defective for replication. On the other hand, HIV-1_{K71R} replicated to a higher extent than the WT virus (Fig. 4B). Thus, a conserved K71R substitution in IN that reduced its affinity for SNF5/Ini1 resulted in an increased viral infectivity.

Transient overexpression of SNF5/Ini1 in A3.01 cells reduced by 68% the replication of a wild-type HIV-1 at 24 h postinfection. In comparison, HIV-1_{K71R} was partially resistant to this inhibition (50% inhibition) (Fig. 4C). It should be noticed that K71R mutation only partially reduces by 2-fold the interaction between SNF5/Ini1 and IN (Fig. 4, A and B). Therefore, a complete resistance to SNF5/Ini1 overexpression should not be expected for the HIV-1_{K71R}.

Virion-associated SNF5/Ini1 Is Not Required for SNF5/Ini1-mediated Inhibition of Early Steps of HIV-1 Replication—Previously, it has been shown that SNF5/Ini1 was incorporated into virions in a Gag-pol precursor-dependent manner, with an integrase to SNF5/Ini1 stoichiometry of ~2:1 *in vitro* (38, 39). Thus, a mutant virus encoding IN K71R is likely to lack SNF5/Ini1 incorporation. To determine the effect of virion-associated SNF5/Ini1 on early steps of HIV-1 replication, viral stocks were produced in HeLa cells treated with either siRNA SNF5.1 or GL2 as control (data not shown). Depletion of SNF5/Ini1 had no effect on the expression of viral proteins in producer cells (data not shown) and only slightly decreased the level of extracellular particle-associated p24 (data not shown). No differences were found between the infectious titers of viral stocks produced in the presence or absence of SNF5/Ini1 (data not shown). Viral stocks were then used to infect HeLa P4.2 reporter cells treated with either siRNA against SNF5/Ini1 (Fig. 5, lower panel, lanes 2 and 4) or control siRNA (Fig. 5, lower panel, lanes 1 and 3). As shown in Fig. 5, inhibition of SNF5/Ini1 in target HeLa P4.2 cells stimulated NL4-3Δenv_{SNF5/Ini1-} infection to the same extent as was observed for NL4-3Δenv_{SNF5/Ini1+} (2.8- and 3.4-fold, respectively). These results indicate that incorporation of SNF5/Ini1 into the viral particle is unlikely to participate in the mechanisms of SNF5/Ini1 inhibition during early steps of the viral replication cycle.

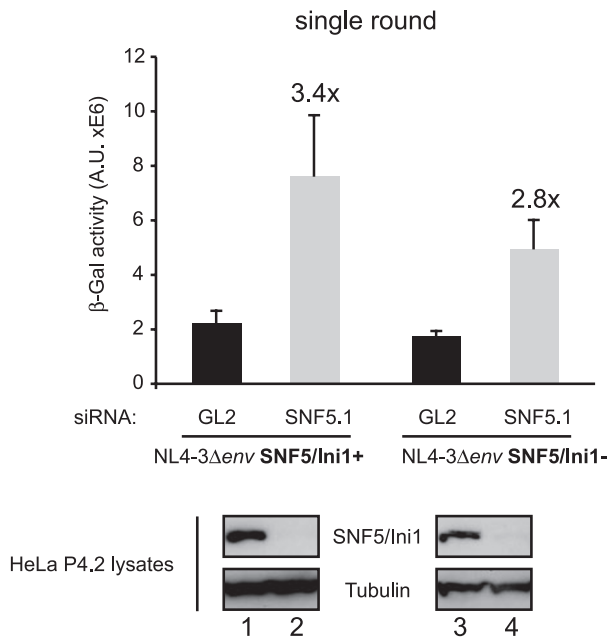


FIGURE 5. SNF5/Ini1 inhibits early steps of HIV-1 replication independently of the presence SNF5/Ini1 within the viral particles. HeLa P4.2 cells were transfected twice with 30 nM SNF5.1 or control (GL2) siRNAs and then infected with either NL4-3Δenv_{SNF5/Ini1+} or NL4-3Δenv_{SNF5/Ini1-} (m.o.i. corresponding to 0.003). 24 h later, the β-galactosidase activity (arbitrary units) was monitored in cell lysates. The mean relative β-galactosidase activities (plus standard errors) obtained from three independent transfection experiments are shown. The -fold stimulation is indicated. SNF5/Ini1 inhibition in these cells was detected 48 h after the second transfection by Western blotting of cell lysates using anti-SNF5/Ini1 antibody, or anti-tubulin antibody as a control.

compared with the wild-type virus. As expected, a virus harboring an integrase with E69G substitution was strongly impaired in its ability to infect HeLa P4.2 cells.

DISCUSSION

SNF5/Ini1 was the first cellular factor identified to interact with IN, but its role in the context of HIV-1 infection remains elusive (8). The results presented here suggest that SNF5/Ini1

SNF5/Ini1 Interferes with Early Steps of HIV-1 Replication

possess an anti-viral activity that interferes with early steps of HIV-1 replication.

Our first approach using RNA interference showed that silencing of SNF5/Ini1 in target cells resulted in an increase in the formation of 2-LTR circle and integrated viral DNA (Fig. 2, C and D). The 2-LTR circles can be the result of the circularization of the viral cDNA by the host cell nonhomologous DNA end-joining pathway and are often used to monitor nuclear import of the PIC (5–7). One could hypothesize that the silencing of SNF5/Ini1 could lead to the stimulation of the nuclear import of the PIC. These results corroborate previous findings suggesting that SNF5/Ini1 participates in an anti-viral cellular response (40). In that study, Turelli *et al.* showed that between 30 min to 6 h postinfection, SNF5/Ini1 along with PML relocalized in the cytoplasm. SNF5/Ini1 and PML export was found to be induced by incoming viral particles (40). SNF5/Ini1 export is likely to be mediated by a masked nuclear export signal that was identified in the rpt1 region of the protein (51). Further studies showed that PML export was independent of SNF5/Ini1, suggesting two separate mechanisms. However, in the same study, no effect on retroviral integration was observed in cells lacking SNF5/Ini1 (41). In our hands, silencing of SNF5/Ini1 using two different siRNA at different concentrations reproducibly stimulated HIV-1 replication (Fig. 1 and data not shown). Thus, an unspecific, off-target effect of our siRNAs is unlikely to explain this observation. However, we noticed that SNF5/Ini1 depletion stimulates HIV-1 replication to a higher extent than retroviral transduction (Fig. 1, B and C). Further experiments will be necessary to explain these differences.

SNF5/Ini1 is part of several multisubunit chromatin remodeling complexes but is also likely to play a role outside the SWI/SNF complexes. Studies in Malignant Rhabdoid Tumor cells have shown that SNF5/Ini1 controls cell proliferation via the Rb cell cycle checkpoint (52). Thus, the stimulation of HIV-1 replication after depletion of SNF5/Ini1 could be an indirect effect of cell proliferation. However, we did not observe changes in the cell cycle of cells transiently knock down for SNF5/Ini1 (see supplemental data).

Our results using RNA interference were further confirmed by the characterization of IN mutants. By performing yeast two-hybrid rebound screenings using SNF5/Ini1 as bait, we mapped the minimal SNF5 binding region between residues 53 and 153 of IN (data not shown). We identified 2 residues within this region, Glu-69 and Lys-71, implicated in SNF5/Ini1 binding (Fig. 3A). Both residues are exposed at the surface of the protein and were not found to be involved in the IN dimer surface (4). Thus, these residues are likely to be available for protein/protein interaction. It has been reported that a virus harboring the double mutation E69A/K71A was replication defective (48). Here, we have shown that this phenotype is due to the substitution of Glu-69 that impaired IN catalytic activity (Fig. 3C). A recent report showed that HIV-1_{K71E} replicated like a WT virus (47). In our study, using a more conservative substitution, HIV-1_{K71R} replication was still increasing at day 18 whereas HIV-1_{WT} peaked at day 12. The level of p24 detected in cells infected with HIV-1_{K71R} was >40-fold higher at day 18 when compared with the WT virus (Fig. 4B). A previous study showed that IN H12Y was defective for SNF5/Ini1 interaction

(38). Furthermore, this residue was described to be involved in the binding of LEDGF/p75 (10). However, mutation of the His-12 residue was shown to markedly reduce the amount of virus-associated reverse transcriptase and IN, so it is possible that the substitution of His-12 perturbs the overall structure of IN (53, 54).

SNF5/Ini1 was found to be specifically incorporated into HIV-1 particles in a Gag-pol precursor-dependent manner (38, 39). A transdominant mutant of SNF5/Ini1 inhibits particle production by directly interacting with IN (38). Despite these observations, the role of virion-associated SNF5/Ini1 remains to be determined. SNF5/Ini1 packaged into virions could be important during the early steps of viral replication in infected cells. However, we found that SNF5/Ini1 depletion in target cells still stimulated the replication of HIV-1 lacking incorporated SNF5/Ini1 (Fig. 5B). These results suggest that the inhibition of early steps of HIV-1 replication by SNF5/Ini1 is not dependent upon the presence of SNF5/Ini1 in virions. Thus, the increase in HIV-1_{K71R} infectivity that we observed is likely to be the consequence of a defect in IN-SNF5/Ini1 interaction at a pre-integration step rather than a defect in SNF5/Ini1 incorporation into viral particles (Fig. 4A).

By interacting with IN, SNF5/Ini1 could destabilize incoming PICs, resulting in the decrease in 2-LTR circular and integrated forms of HIV-1 provirus. It is noteworthy that the SNF5/Ini1 effect on viral replication is similar to the one described for RAD52 (55). RAD52 is a protein involved in homologous recombination, one of the pathways implicated in the repair of double-strand breaks (56). RAD52 was shown to markedly reduce retroviral infection. Inhibition of its expression led to an increase in levels of 2-LTR circles and integrated proviral DNA, a phenotype that is similar to the one we observed after SNF5/Ini1 knock down (55). It was suggested that RAD52 could directly compete with Ku for the binding to cDNA viral ends (55). Thus, one could hypothesize that SNF5/Ini1 interacts with RAD52 and could participate in the displacement of Ku from incoming viral cDNA. Interestingly, a recent study in yeast showed that SWI/SNF participates in homologous recombination repair at, or just preceding, the strand invasion step (57). These results suggest that the antiviral effect of SNF5/Ini1 could be mediated by the homologous recombination repair machinery.

Acknowledgments—We thank Linos Vandekerckhove and Zeger Debysier for the generous gift of the enhanced GFP HIV vector.

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