Interaction of Human Immunodeficiency Virus Type 1 Integrase with Cellular Nuclear Import Receptor Importin 7 and Its Impact on Viral Replication*

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Similar to all other viruses, human immunodeficiency virus type 1 (HIV-1) depends heavily on cellular factors for its successful replication. In this study we have investigated the interaction of HIV-1 integrase (IN) with several host nuclear import factors using co-immunoprecipitation assays. Our results indicate that IN interacts specifically with host importin 7 (Imp7) in vivo, but does not interact with importin 8 (Imp8) or importin α (Rch1). In contrast, another HIV-1 karyophilic protein MAP17, which is capable of binding Rch1, fails to interact with Imp7, suggesting that IN and MAP17 may interact with different cellular pathways during HIV-1 replication. Genetic analysis revealed that the C-terminal domain of IN is the region responsible for interaction between IN with Imp7, and an IN mutant (K240A,K244A/R263A,K264A) disrupted the Imp7 binding ability of the protein, indicating that both regions (235WKGPAKLLWKG and 262RRKAK) within the C-terminal domain of IN are required for efficient IN/Imp7 interaction. Using a vesicular stomatitis virus G glycoprotein pseudotyped HIV single-cycle replication system, we showed that the IN/Imp7 interaction-deficient mutant was unable to mediate viral replication and displayed impairment at both viral reverse transcription and nuclear import steps. Moreover, transient knockdown of Imp7 in both HIV-1 producing and target cells resulted in a 2.5–3.5-fold inhibition of HIV infection. Altogether, our results indicate that HIV-1 IN specifically interacts with Imp7, and this viral/cellular protein interaction contributes to efficient HIV-1 infection.

To carry out a successful infection, human immunodeficiency virus type 1 (HIV-1)8 takes advantage of various host cellular proteins and cellular pathways. The interaction between cellular proteins and viral components takes place during various steps of the HIV-1 life cycle, including viral DNA nuclear import. The most striking feature of HIV-1 is its ability to replicate in non-dividing cells. This feature depends on the ability of the virus to transport its cDNA, as part of a large preintegration complex (PIC), from the cytoplasm to the nucleus by an active and energy-dependent process (1–3). However, the mechanism by which the PIC translocates across the nuclear membrane into the nucleus of non-dividing cells is still not fully understood. It has been shown that three HIV-1 PIC-associated proteins including MAP17, IN, and Vpr possess karyophilic properties, and contribute to nuclear translocation of viral PICs. This action is accomplished through their interactions with karyophilic cellular proteins, thereby directing the PIC through the nuclear pore complex (4–10). In addition, a cis-acting element named the central DNA flap, which is located in the 3′ region of the pol gene sequence, was also shown to contribute to HIV-1 nuclear import in both dividing and non-dividing cells (11–14).

Nuclear import of proteins in mammalian cells can be mediated by several distinct pathways. The importin α/β heterodimer mediates nuclear import of proteins harboring a classical nuclear localization signal, which either contains a cluster of basic amino acids or two basic clusters separated by 10–20 amino acids (bipartite nuclear localization signal) (for reviews see Refs. 15 and 16). Also, importin β (Impβ) was shown to bind to and import HIV-1 proteins, such as HIV-1 Tat, Rev, and HTLV Rex, independently of importin α (Impα) (17–21). Similarly, transportin, an Impβ-related receptor, imports its substrates (heterogeneous nuclear ribonucleoproteins) by directly binding to the glynine-rich M9 domain of the protein (22, 23). Based on the similarity to Impβ, several other nuclear import factors, including Imp7 and Imp8, have also been identified (24). Imp7 is one of several cellular importins that bind to and mediate nuclear import of ribosomal proteins in mammalian cells, and it was also found to translocate other proteins, such as glucocorticoid receptor and histone H1 into the nucleus (18, 25–27). In the case of histone H1, Jakel et al. (27) have demonstrated that two receptors, Impβ and Imp7, enhanced yellow fluorescent protein; RT, reverse transcription; GFP, green fluorescence protein; scRNA, scrambled RNA; β-Gal, β-galactosidase; wt, wild type; GST, glutathione S-transferase; luc, luciferase.
form a heterodimer, and this complex is the functional unit required for nuclear import of histone H1. Imp8 also contributes to the nuclear import of signal recognition particle protein 19 (28). However, whether and how these cellular proteins contribute to the nuclear import of HIV-1 PIC during the early stage of viral replication remains undefined.

HIV-1 integrase (IN) is a 32-kDa protein that plays a key role in viral DNA integration into the host chromosome. In addition, this viral protein has also been shown to contribute to other steps during the early stage of viral replication, including reverse transcription (29, 30) and viral DNA nuclear import (7, 10, 31, 32). Even though IN has been well documented to possess karyophilic properties (7, 10, 33–35), the mechanism by which HIV-1 IN contributes to nuclear import of the viral PIC is still not fully understood. Some previous studies have showed that IN is capable of binding to Impα in vitro binding assays (7, 36, 37), but the in vitro nuclear import assay results concerning whether Impα plays a role in the nuclear translocation of IN and/or HIV-1 DNA, is still controversial (33, 37, 38). A cellular component, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75), was initially implicated in contributing to the nuclear translocation of HIV-1 (39, 40). However, the following study came to the conclusion that the interaction of IN with LEDGF/p75 may not be required for IN nuclear localization (41). Several studies have further identified that LEDGF/p75 is important for tethering IN as well as the viral PIC to chromosomal DNA, and contributes to controlling the location of HIV DNA integration (41–43). In attempts to search for other cellular factor(s) involved in HIV-1 nuclear import, Fassati et al. (38) have reported that Imp7 contributes to HIV-1 PIC nuclear import by using in vitro nuclear import assays. Their study also showed that small interfering RNA (siRNA) mediated Imp7-knockdown inhibited HIV-1 replication (38). In addition, their in vitro binding assay showed that recombinant IN could pull down several cellular nuclear import receptors including Impo, Impβ, Imp7, and transportin from HeLa cell lysates, suggesting that the action of Imp7 in HIV-1 PIC nuclear import may be through its binding to HIV-1 IN (supplementary materials in Ref. 38). However, a recent study by Zielske and Stevensen (44) did not reveal the impact of Imp7 knockdown on HIV-1 and SIV nuclear import in macrophages. Therefore, the functional role of Imp7 during HIV-1 replication remains to be defined.

In this study, we have investigated the interaction of HIV-1 IN with several cellular importins by using a cell-based co-immunoprecipitation assay. Our results indicate that HIV-1 IN specifically interacts with Imp7, but not with Impα (Rch1) or Imp8, and this IN/Imp7 interaction takes place in HIV-1-infected T cells. We also showed that another HIV-1 karyophilic protein MAP17, which is capable of binding Rch1, was unable to interact with Imp7. Our mutagenic analysis demonstrated that two regions (235WKGPAKLLWKG and 262RRKAK) in the C-terminal domain of IN are critical for its Imp7 binding ability. To further elucidate the contribution of the IN/Imp7 interaction to HIV-1 replication, we have shown that an Imp7-binding defective IN mutant virus lost infectivity and displayed defects during both reverse transcription and nuclear import. Moreover, our experiments revealed that HIV-1 produced from Imp7-depleted cells, exhibited a 2.5–3.5-fold reduced infection in Imp7-knockdown susceptible cells.

**EXPERIMENTAL PROCEDURES**

**Construction of Different Viral and Cellular Protein Expressors**—To generate a CMV-YFP-IN fusion expressor, the full-length wild-type HIV-1 IN cDNA was amplified from HIV-1 HxBru provirus (45) by PCR using a 5′-BglII primer (5′-GCCAGATCTTTTCTAGATGGAATAAAG-3′) and a 3′-BamHI primer (5′-CTAAACGGAATCTAGTTCTAA-3′). The amplified HIV-1 IN fragment was cloned in-frame at the 3′ end of the EYFP cDNA in a pYEFP-C1 vector (Clontech). The CMV-IN-YFP and CMV-IN(50–288)-YFP expressors used in the study were previously described (32). To construct different CMV-YFP-IN deletion mutants, cDNA fragments encoding amino acids 1–212 and 1–240 of IN were generated using PCR with 5′-BglII and 3′ primers (5′-CAATTCCCCGGGTGTATGTCTGTGTTGC-3′; 5′-CCAGACCGGGTTGCTGTGCTTCCATTCCA-3′), and was inserted into the pYEFP-C1 vector at BglII and Xmal sites. Different IN substitution mutants were generated by a two-step PCR-based method (46), using a 5′-BglII primer, 3′-Xhol primer, and complementary primers containing the desired mutations. The amplified IN cDNAs harboring specific mutations were then cloned into pYEFP-C1 vector. To generate HIV-1 provirus NL4.3-BruΔBgl/Luc, the sequence from the ApaI to Sall site (nucleotides 1556–5329, +1 = start of NL4.3 initiation of transcription) in a RT/IN/Env defective HIV-1 provirus NLucΔBgl/ΔR (32) was replaced by the corresponding sequences of HIV-1 provirus HxBru (45). The genotype of this molecular clone is the 5′ long terminal repeat gag + pol + vif + vpr + tat + rev + vpu + env − nef − 3′ long terminal repeat. To construct provirus HxBru containing a HA tag at the C terminus of IN, a DNA sequence encoding amino acid YPYDVPDYASLG was inserted at the 3′ end of IN encoding sequence by a two-step PCR method, in a natural Apal/Sall fragment derived from HxBru (13). Then, this PCR-generated Apal/Sall fragment was then inserted back to the provirus HxBru and the constructed provirus was named HxBru-IN-HA.

The pGEX-Imp7 and pGEX-Imp8 plasmids encoding for *Xenopus* Imp7 and human Imp8 cDNAs were generously provided by Dr. Yamamoto (26), and used as PCR templates for constructing CMV-T7-Imp7 and CMV-T7-Imp8 plasmids. The cDNA encoding Rch-1 was amplified from pET-21-Rch1. The amplified Imp7, Imp8, and Rch-1 fragments were digested with BamHI and NotI and cloned at the 3′ end of IN encoding sequence derived from HxBru (13). Then, this PCR-generated Apal/Sall fragment was then inserted back to the provirus HxBru and the constructed provirus was named HxBru-IN-HA.
HIV-1 Integrase Interaction with Importin 7

Antibodies and Chemicals—Antibodies used in immunoprecipitation or Western blot are as follows. The purified rabbit anti-GFP polyclonal antibody and mouse monoclonal anti-GFP antibodies were obtained from Molecular Probes Inc. The mouse anti-T7 antibody was obtained from Novagen Inc. (Darmstadt, Germany). The rabbit anti-human Imp7 antibody was kindly provided by Dr. A. Fassati (38). The rabbit anti-IN antibodies (catalog number 757) and the purified recombinant HIV-1NL4.3 IN protein (catalog number 9420) were obtained through the AIDS Research Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. The human anti-HIV serum, mouse anti-HA, and anti-Myc antibodies were kindly provided by Dr. Eric A. Cohen. The ECL™ horseradish peroxidase-conjugated donkey anti-rabbit IgG, and the sheep anti-mouse IgG were purchased from Amersham Biosciences. The Western blot detection ECL kit was purchased from PerkinElmer Life Science (Boston, MA). CHAPS was purchased from Sigma.

Cell Culture and Transfection—Human embryonic kidney 293T cells and HeLa-β-gal-CD4/CCR5 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, and 1% penicillin and streptomycin. The CD4+ C8166 T cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. DNA transfection in 293T cells was performed with a standard calcium phosphate DNA precipitation method. After 48 h of transfection, cells were harvested and ready for different experiments.

IN/Imp7 Binding Assays Using Immunoprecipitation (IP) and Western Blot—To test protein expression and the protein/protein interaction in mammalian cells, 293T cells were transfected or co-transfected with corresponding protein expression plasmids. After 48 h of transfection, cells were lysed with CHAPS lysis buffer (199 medium containing 0.5% CHAPS and a protease inhibitor mixture (Roche)) on ice for 30 min, and clarified by centrifugation at 13,000 g for 30 min at 4 °C. Then, the supernatant was subjected to immunoprecipitation with rabbit anti-GFP or the corresponding antibody. Immunoprecipitants were resolved by 10% SDS-PAGE, followed by Western blot using mouse anti-T7 or mouse anti-GFP antibodies, respectively. Also, the total T7-tagged protein expression in cell lysates was sequentially immunoprecipitated with mouse anti-T7 antibody, followed by Western blot using the same antibody.

To test the interaction of HIV-1 IN with endogenous Imp7, 293T cells were mock-transfected, YFP transfected or transfected with IN-YFP expression plasmids, and the same IP and Western blot protocols were used as described above, except using rabbit anti-imp7 antibody to check the bound endogenous Imp7. Meanwhile, non-transfected 293T cell lysate was loaded directly in SDS-PAGE as a positive control. To test the IN/Imp7 interaction in HIV-1-infected CD4+ T cells, the CD4+ C8166 cells were infected with HIV-1 (vIN-HA), which was produced in 293T cells transfected with provirus HxBru-IN-HA. After 48–72 h post-infection, C8166 cells were lysed with 0.5% CHAPS lysis buffer and IP with anti-HA antibody. Immunoprecipitants were then resolved by 10% SDS-PAGE followed by Western blot using anti-imp7 antibody for detecting bound Imp7. In parallel, the normal C8166 T cell lysate was loaded directly in SDS-PAGE as positive control. Meanwhile, the IN-HA expression was detected with rabbit anti-IN antibody.

In Vitro Binding Studies—To produce GST and GST-Imp7 proteins, Escherichia coli BL21 cells transformed with pGEX-4T-GST or pGEX-4T-GST-Imp7 plasmids were cultured in LB medium (0.1 mg/ml ampicillin). Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h at 37 °C. Bacteria were harvested, and suspended in 35 ml of ice-cold column buffer, and broken by sonication (five 30-s pulses at 100 watts, Sonics & Materials, Inc.). The resulting lysates were centrifuged for 30 min at 13,000 × g, and passed through a glutathione-Sepharose 4B column (Amersham Biosciences). After being washed by column buffer, the bound GST and GST-Imp7 proteins were eluted by glutathione buffer (100 mM reduced glutathione (Roche), 120 mM NaCl, 100 mM Tris-HCl, pH 8.5). Finally, the eluted protein was dialyzed in phosphate-buffered saline to remove glutathione.

For in vitro binding experiments, equal amounts of recombinant GST or GST-Imp7 protein were incubated with a recombinant HIV-1 IN in 199 medium containing 0.1% CHAPS, for 2 h at 4 °C. Then, 100 µl of glutathione-Sepharose 4B beads were added and incubated for an additional hour. The beads were washed and the bound proteins were eluted with 50 mM glutathione, and loaded onto a 12.5% SDS-PAGE for Western blot analysis with rabbit anti-IN antibodies.

Transient Knockdown of Imp7 in 293T and HeLa-β-GalCD4/CCR5 Cells—293T and HeLa-β-Gal-CD4/CCR5 cells were plated at 2 × 10⁵ cells/well in 6-well plates, and transfected the next day with 100 pmol of Imp7-specific siRNA duplex (IPO7-HSS116173) with Lipofectamine™ RNAiMax reagent (Invitrogen). After 18 h of the first transfection, another Imp7 siRNA duplex (IPO7-HSS116174) was transfected again into the cells. These two Imp7-siRNA duplexes (Stealth RNAi), IPO7-HSS116173 and IPO7-HSS116174, were synthesized by Invitrogen Inc. The targeting sequences correspond to Imp7 mRNA nucleotides 1990–2013 (5′-UAAGCAGAUUUCGUCAACGCUUGG-3′), and to Imp7 mRNA nucleotides 610–633 (sense 5′-AUGCGUUGCUAGCGCAUGG-3′). In parallel, transfection of scramble (sc) RNA (purchased from Santa Cruz Biotechnology) was used as control. After 48 and 72 h of transfection, cells were used for different HIV-1 provirus transfection and viral infections, respectively.

Virus Production and Infection—To test the effect of the Imp7-binding defective mutant on HIV replication, a vesicular stomatitis virus G (VSV-G) glycoprotein pseudotyped single-cycle replicating virus was produced in 293T cells, as described previously (32). Briefly, 293T cells were transfected with an RT/IN/Env-deleted HIV-1 provirus NLucΔBglΔRI, each CMV-Vpr-RT-IN (wt/mutant) expressor and a VSV-G expressor. Introduction of IN mutations into the CMV-Vpr-RT-IN expressor was done through a PCR-based method as described previously (32). To produce viruses from Imp7-siRNA- or scRNA-transfected cells, the same amount of cells were transfected with Imp7 siRNA or scRNA twice (at 0 and 18 h), and after 36 h of the first transfection cells were re-plated. After an additional 12 h, cells were transfected with different HIV-1 proviruses. After 48 h of provirus transfection, virus was collected...
following Western blotting using the same antibody (lanes 4 and 5).

C, the in vivo co-IP assay. CMV-IN-YFP was co-transfected with plasmid T7-Imp7 (lane 3) or T7-Imp8 (lane 4) into 2 × 10⁵ 293T cells. A control, CMV-IN-YFP also was co-transfected with each importin expressing plasmid (lanes 1 and 2). After 48 h of transfection, cells were lysed with 0.5% CHAPS buffer and immunoprecipitated with rabbit anti-GFP antibody. The immunoprecipitated complexes were resolved by 12.5% SDS-PAGE and immunoblotted with either mouse anti-T7 antibody (upper panel) or mouse anti-GFP antibody (middle panel). The unbound T7-Imp7 and T7-Imp8 were also checked by sequential IP with anti-T7 antibody followed by the Western blot (WB) with the same antibody (lower panel).

FIGURE 1. Interaction of HIV-1 IN and importin 7. A, schematic representation of constructs of IN-YFP, T7-Imp7 and -Imp8. For IN-YFP, a full-length wild-type HIV-1 IN was fused in-frame to the N terminus of EYFP. For T7-Imp7 and Imp8, a T7 tag (9 amino acids) was fused in-frame to the N terminus of Imp7 and Imp8. B, expression of IN-YFP and T7-Imp7 and T7-Imp8. Cell lysates from about 6 × 10⁵ 293T cells transfected with CMV-YFP, CMV-IN-YFP, or the indicated importin expressors were analyzed by IP with rabbit anti-GFP antibody followed by Western blotting using mouse anti-GFP antibody (lanes 1–3) or IP with mouse anti-T7 antibody followed by Western blotting using the same antibody (lanes 4 and 5). C, the in vivo co-IP assay. CMV-IN-YFP was co-transfected with plasmid T7-Imp7 (lane 3) or T7-Imp8 (lane 4) into 2 × 10⁵ 293T cells. A control, CMV-IN-YFP also was co-transfected with each importin expressing plasmid (lanes 1 and 2). After 48 h of transfection, cells were lysed with 0.5% CHAPS buffer and immunoprecipitated with rabbit anti-GFP antibody. The immunoprecipitated complexes were resolved by 12.5% SDS-PAGE and immunoblotted with either mouse anti-T7 antibody (upper panel) or mouse anti-GFP antibody (middle panel). The unbound T7-Imp7 and T7-Imp8 were also checked by sequential IP with anti-T7 antibody followed by the Western blot (WB) with the same antibody (lower panel).

HIV-1 Reverse Transcribed and Nuclear Imported DNA Detection by PCR and Southern Blotting—C8166 T cells were infected with equal amounts of the VSV-G pseudotyped IN wt or mutant viruses for 2 h, washed with phosphate-buffered saline, and cultured in RPMI medium. At 12 or 24 h post-infection, an equal number (1 × 10⁶ cells) of cells were collected, and processed for detecting total viral DNA synthesis or nuclear- and cytoplasm-associated viral DNA by PCR and Southern blotting, as described previously (32).

RESULTS

HIV-1 IN Interacts with Imp7 but Not with Imp8—To investigate the interaction of HIV-1 IN with different cellular nuclear import factors, we first tested the interaction of HIV-1 IN with Imp7 and Imp8, by using a cell-based co-immunoprecipitation (co-IP) assay. The SVCMV-T7-Imp7 and T7-Imp8 expressing plasmids were constructed by inserting Imp7 and Imp8 cDNAs, into a SVCMV-T7 vector at the 3' end of a T7 tag encoding sequence (Fig. 1A), as described under “Experimental Procedures.” Also, a previously described HIV-1 IN-YFP fusion protein expressor CMV-IN-YFP (32), and a CMV-YFP expressor were used in the study and shown in Fig. 1A. First, protein expression was checked by transfecting each of these plasmids into 293T cells, and processed using anti-GFP or anti-T7 IP, followed by Western blot with corresponding antibodies. Results showed that IN-YFP and YFP were detected at positions 58 and 27 kDa, respectively (Fig. 1B, lanes 2 and 3), whereas T7-Imp7 and T7-Imp8 were at positions that ranged between 110 and 130 kDa (Fig. 1B, lanes 4 and 5).

To test whether IN-YFP could bind to different importin, the YFP or IN-YFP expressor was co-transfected with each importin expressor in 293T cells, as indicated in Fig. 1C. After 48 h, cells were lysed with CHAPS lysis buffer (199 medium containing 0.5% CHAPS), and immunoprecipitated using rabbit anti-GFP antibody. Precipitated complexes were run on an SDS-PAGE, followed by Western blot with anti-T7 antibody (Fig. 1C, upper panel). Interestingly, results revealed that, whereas the YFP protein did not co-precipitate with any importin (Fig. 1C, upper panel, lanes 1 and 2), the IP of IN-YFP specifically co-pulled down T7-Imp7 (Fig. 1C, lane 3, but not T7-Imp8 (Fig. 1C, lane 4). Meanwhile, the immunoprecipitated IN-YFP and YFP in each sample, respectively, were checked by anti-GFP Western blot, and similar levels of each protein were detected (Fig. 1C, middle panel, lanes 3 and 4). To rule out the possibility that the co-precipitated T7-Imp7 was due to differential levels of importin expression in each transfection sample, the cell lysates were processed using sequential IP with anti-T7

and concentrated from the supernatant using ultracentrifugation. Virus titers were quantified using HIV-1 p24 Antigen Capture Assay Kit (purchased from the NCI-Frederick AIDS Vaccine Program).

To infect CD4+ T cells, equal amounts of virus (adjusted by virion-associated p24 level) were incubated with C8166 T cells at 37 °C for 4 h. At different time points post-infection, 1 × 10⁶ cells from each sample were collected, and lysed with 50 μl of luciferase lysis buffer (Fisher Scientific Inc). 10 μl of cell lysate was subjected to the luciferase assay by using a TopCount®NXT™ Microplate Scintillation & Luminescence Counter (Packard, Meriden), and the luciferase activity is valued as relative luciferase units. Each sample was analyzed in duplicate, and the average deviation was calculated. To test the effect of Imp7 knockdown on HIV-1 infection, at 72 h after being transfected with Imp7-siRNA or scRNA, HeLa-β-GalCD4/CCR5 cells were infected with equal amounts of different viruses in the presence of DEAE-dextran (20 μg/ml). At 48 h post-infection, HIV-1 infection was monitored by measurement of the luciferase activity level and/or the amount of β-galactosidase positive cells, as described previously (47).
HIV-1 Integrase Interaction with Importin 7

A. Mixture of cells

|                | Mock-transfected cells | T7-imp7 | IN-YFP | T7-imp7 + INYFP |
|----------------|------------------------|---------|--------|-----------------|
| IP: anti-GFP   | WB: anti-imp7          | 120     | 99     | 120             |
| IP: anti-T7    | WB:                    |         |        |                 |

B. 293T cells

|                | IP: anti-GFP           | WB: anti-imp7 | -T7-imp7 | IN-YFP | Imp7 (Bound) |
|----------------|------------------------|---------------|----------|--------|--------------|
| W:             | lane 1: anti-imp7      | 120           | 99       |        |              |
| D:             | lane 2: anti-Imp7       |               |          |        |              |

C. C8166 cells

|                | IP: anti-HA or anti-Myc | WB: anti-imp7 | WB: anti-IN | WB: anti-HA |
|----------------|------------------------|---------------|-------------|-------------|
| W:             | lane 1: anti-imp7      | 100           | 100         | 100         |
| D:             | lane 2: anti-imp7      |               |            |             |

D. HIV-1 IN

|                | GST: Imp7              | GST-imp7      | GST-imp7   | IN (dimer) |
|----------------|------------------------|---------------|------------|------------|
| W:             | lane 1: anti-Imp7      | 120           | 99         |            |
| D:             | lane 2: anti-Imp7      |               |            |            |

FIGURE 2. HIV-1 IN interacts with endogenous Imp7 and the IN-Imp7 interaction takes place in the cells and in vitro. A, IN-Imp7 interaction in the cells. The IN-YFP and T7-Imp7 plasmids were co-transfected (lane 2) or transfected individually (lane 3) into 293T cells. After 48 h, cells were mixed accordingly, lysed, and analyzed by Western blot using rabbit anti-IN antibody. Mean-
interaction of IN and Imp7 in vitro, similar amounts of purified GST and GST-Imp7 were incubated with a purified recombinant HIV-1 IN in 199 medium containing 0.1% CHAPS for 2 h at 4 °C, followed by an additional 1-h incubation with glutathione-Sepharose 4B beads. Then, the bound protein complex was eluted out with 100 mM glutathione, and loaded onto a 12.5% SDS-PAGE gel, followed by Western blot analysis with anti-IN antibodies. Results showed that the purified HIV-1 IN, in both of dimer and monomer forms, was able to specifically interact with GST-Imp7, but not with GST (Fig. 2D, right panel). Thus, the binding of IN to Imp7 may be through a direct protein/protein interaction.

Differential Binding Ability of HIV-1 IN and MAp17 to Imp/Imp7—The importin α/β nuclear translocation pathway has been implicated in assisting with HIV-1 nuclear import (6, 7). Several HIV-1 proteins, including MAp17, Vpr, and IN have been shown to interact with Imp/Imp7 in vitro binding assays (6, 7, 36, 38, 48). In this study, we attempted to test whether HIV-1 IN could interact with Rch1, a member of the human importin α family (49), by using a co-IP assay. A T7-tagged Rch1 expressing plasmid (CMV-T7-Rch1), and an HIV-1 MAp17G2A mutant-YFP fusion protein expressing plasmid (CMV-MAp17G2A-YFP) were constructed. In MAp17G2A-YFP, glycine, the second amino acid, was replaced by alanine, and this MAp17 mutant was previously shown to be capable of binding to Rch1 in a cell-based co-IP system (6). After IN-YFP or MAp17G2A-YFP were co-expressed with T7-Rch1 in 293T cells, their interaction with Rch1 was analyzed using the same co-IP and Western blot protocols, as described in Fig. 1. Consistent with a previous report (6), MAp17G2A-YFP was shown to be able to bind to T7-Rch1 (Fig. 3A, lane 4). However, IN-YFP did not show any interaction with T7-Rch1 (Fig. 3A, lane 3). In contrast, whereas T7-Imp7 co-precipitated with IN-YFP, no T7-Imp7 was detected in the immunoprecipitated MAp17G2A-YFP sample (Fig. 3B, compare lane 4 to 3). These results suggest that HIV-1 IN and MAp17 may interact with different cellular nuclear import factors during HIV-1 replication.
Delineation of Region(s) of HIV-1 IN Required for Its Interaction with Imp7—To delineate which region(s) within HIV-1 IN is required for its Imp7 binding, we first tested a previously described IN N-terminal deletion mutant (CMV-IN50–288-YFP) expressor (32) (Fig. 4A) for Imp7 binding. The co-IP analysis revealed that, similar to the IN-YFP, the IN50–288-YFP bound efficiently to T7-Imp7 as well (Fig. 4B, compare lane 5 to 4), indicating that the N-terminal domain of IN is not required for the IN7-Imp7 interaction.

To test the core domain and the C-terminal domain of IN for their contribution toward Imp7 binding, we constructed three YFP-IN expressors, including CMV-YNwT and two IN C-terminal deletion mutants (CMV-YFP-IN1–212 and CMV-YFP-IN1–240) (Fig. 4A). For the CMV-YNwT expressor, the PCR-amplified HIV-1 IN full-length cDNA was placed in frame at the 3′ end of the YFP cDNA, whereas for CMV-YFP-IN1–212 and CMV-YFP-IN1–240, sequences encoding for the last 76 and 48 amino acids of IN were removed, respectively. Expression of each YFP-IN fusion protein along with its ability to bind Imp7 was tested in 293T cells, by co-transfecting each YFP-IN fusion protein expressor with the T7-Inp7 plasmid. The position of each immunoprecipitated and co-precipitated proteins were indicated on the right side of the gel. WB, Western blot.

Effect of Imp7-binding Defective IN Mutant on HIV-1 Infection in CD4+ C8166 T Cells—Given that IN mutant INKKRRK lost its Imp7 binding ability, we next examined the effect of this IN mutant on HIV-1 replication. To do so, the INKKRRK mutant was first introduced into a CMV-Vpr-RT-IN expressor. Then, the VSV-G pseudotyped HIV-1 single cycle replicating virus (vKKRK) was produced in 293T cells by co-transfection with CMV-Vpr-RT-IN expressor, as described previously (32). In parallel, the VSV-G pseudotyped wild type virus (vINwt) and IN class I mutant D64E virus (vD64E) were also produced as controls. After each virus stock was harvested, the trans-incorporation of RT and IN as well as the GAG composition in the viral particle was analyzed using Western blot with human anti-HIV positive serum. Results showed that similar
amounts of RT, IN, and Gagp24 were detected in each virus preparation (Fig. 6A). Then, equal amounts of each virus stock (as adjusted by amounts of HIV-1 Gagp24) were used to infect CD4+ C8166 cells. At different time intervals, the luciferase activity in equal amounts of cells was measured, as shown in Fig. 6B. Because D64E mutant virus (vD64E) is unable to mediate viral DNA integration, its infection induced very low luciferase activity, which only reached 0.8% of the luciferase activity level detected from the wt virus infection (Fig. 6B). Interestingly, the luciferase activity of the vKKRK virus infection was considerably lower than that of the D64E mutant virus at different time points (Fig. 6B), indicating that the vKKRK virus lost its replication ability in CD4+ C8166 cells.

To test at which step the Imp7-binding defect mutant virus infection was affected, the cytoplasm- and nucleus-associated viral DNA levels were analyzed at 24 h post-infection, using semi-quantitative PCR and Southern blot. For the vKKRK virus infection, the level of total viral DNA (including the cytoplasm- and nucleus-associated viral DNA levels) was reduced by ∼60%, compared with the total viral DNA level detected from the wt virus infection (Fig. 6C, upper panel, compare lanes 5 and 6 to lanes 1 and 2, and D, left panel). Moreover, results revealed that for the wt and vD64E virus infections, ∼73 and 77% of viral DNA was nucleus-associated (Fig. 6, C, upper panel, lanes 1–4, and D, right panel). However, during vKKRK infection, only 44% of viral DNA was nucleus-associated (Fig. 6, C, upper panel, lanes 5 and 6, and D, right panel). The integrity of the fractionation procedure was also validated by detection of β-globin DNA, which was found solely in the nucleus, and levels of this cellular DNA were similar in each nuclear sample (Fig. 6C, lower panel). Taken together, all of these results indicate that the Imp7-binding defect mutant virus vKKRK is unable to replicate in C8166 cells, and displays impaired viral reverse transcription and nuclear import.
Effect of Imp7 Knockdown on HIV-1 Replication—To further elucidate the contribution of Imp7 to HIV-1 replication, we also investigated the effect of siRNA-mediated Imp7 knockdown on HIV-1 replication. First, we tested the efficiency of Imp7 knockdown. Imp7-siRNA (100 pmol) was introduced into 293T and HeLa-β-Gal-CD4/CCR5 cells once a day for 2 days (Fig. 7A), and at different time intervals, equal amounts of cells (0.5 × 10^6 cells) were collected and monitored for Imp7 expression. Western blot results indicated that Imp7 protein expression was progressively decreased over the course of the experiments. At 48 h following the first Imp7-siRNA transfection, the Imp7 protein level was reduced to ∼30%, and at 96 h, the level of Imp7 expression was reduced to ∼10% in both 293T and HeLa-β-Gal-CD4/CCR5 cells (Fig. 7B).

Next, we tested the effect of Imp7 knockdown on HIV-1 infection. To avoid the possibility that Imp7 might have an effect on the late stage of viral replication, and/or be packaged into viral particles and thus playing a role in subsequent viral infection, we first produced VSV-G-pseudotyped HIV-1 virus containing the luciferase gene (sc-virus and si-virus). Both viruses were then used to infect HeLa-β-Gal-CD4/CCR5 cells that have been treated with Imp7 siRNA or scRNA for 72 h. After 48 h post-infection, the luciferase activity was measured. The results are representative for three independent experiments.

**FIGURE 7.** siRNA-mediated silencing of Imp7 inhibits HIV-1 infection. A, experimental design for the duration of siRNA treatments and HIV-1 transfection and infection. B, siRNA-mediated silencing of Imp7 in 293T and HeLa-β-Gal-CD4/CCR5 cells. Cells were transfected with 20 nm siRNA at 0 and 18 h. After 48, 72, and 96 h post-initial transfection, the Imp7 expression levels in each cell line were verified by Western blot with anti-Imp7 antibody (upper panel). Meanwhile, the expression of α-tubulin was also verified (lower panel). C, 293T cells were treated with scRNA or siImp7 once a day for 2 days and used to produce VSV-G-pseudotyped HIV-1 virus containing the luciferase gene (sc-virus and si-virus). Both viruses were then used to infect HeLa-β-Gal-CD4/CCR5 cells that have been treated with Imp7 siRNA or scRNA for 72 h. After 48 h post-infection, the luciferase activity was measured. The results are representative for three independent experiments. D, scRNA- or siImp7-treated HeLa-β-Gal-CD4/CCR5 cells were infected with the wt HxBru virus produced from scRNA- or siImp7-treated HeLa cells. After 48 h post-infection, viral infection was evaluated by MAGI assay. The results are representative for two independent experiments.

Effect of Imp7 Knockdown on HIV-1 Replication—To further elucidate the contribution of Imp7 to HIV-1 replication, we also investigated the effect of siRNA-mediated Imp7 knockdown on HIV-1 replication. First, we tested the efficiency of Imp7 knockdown. Imp7-siRNA (100 pmol) was introduced into 293T and HeLa-β-Gal-CD4/CCR5 cells once a day for 2 days (Fig. 7A), and at different time intervals, equal amounts of cells (0.5 × 10^6 cells) were collected and monitored for Imp7 expression. Western blot results indicated that Imp7 protein expression was progressively decreased over the course of the experiments. At 48 h following the first Imp7-siRNA transfection, the Imp7 protein level was reduced to ∼30%, and at 96 h, the level of Imp7 expression was reduced to ∼10% in both 293T and HeLa-β-Gal-CD4/CCR5 cells (Fig. 7B).

Next, we tested the effect of Imp7 knockdown on HIV-1 infection. To avoid the possibility that Imp7 might have an effect on the late stage of viral replication, and/or be packaged into viral particles and thus playing a role in subsequent viral infection, we first produced VSV-G-pseudotyped HIV-1 (NL4.3-BruΔBgl/luc+) from Imp7-siRNA- or scRNA-transfected 293T cells. The Imp7 protein expression in siRNA transfection cells was <10% (at 96 h of siRNA transfection) when the viruses were collected. Then, viruses (si-virus and sc-virus) produced from Imp7-siRNA- or scRNA-transfected 293T cells were normalized by HIV Gagp24 levels, and used to infect scRNA- and siRNA-treated HeLa-β-Gal-CD4/CCR5 cells (target cells) (Fig. 7A). Results in Fig. 7C shows that there is no significant difference in luciferase activity detected in scRNA- and siRNA-treated target cells after being infected with sc-virus (Fig. 7C, bars 1 and 2) or in the scRNA-treated cells being infected by si-virus (Fig. 7C, bar 3). However, when siRNA-treated HeLa-β-Gal-CD4/CCR5 cells were infected with si-virus, the luciferase activity was reduced to ∼37% of the wt infection level (Fig. 7C, compare bar 4 to 1).

These observations were further extended to HIV-1 envelope-mediated viral infection. HIV-1 envelope competent siHxBru and scHxBru viruses were produced in Imp7-siRNA and scRNA-treated HeLa-β-Gal-CD4/CCR5 cells by transfecting with HIV-1 HxBru provirus (45), and used to infect the Imp7-siRNA and scRNA-treated HeLa-β-Gal-CD4/CCR5 cells,
at 72 h post-transfection. The numbers of β-Gal positive cells were evaluated by MAGI assay at 48 h post-infection. As expected, when Imp7-siRNA-treated HeLa-β-Gal-CD4/CCR5 cells were infected with si-virus, the β-Gal positive cell level was significantly reduced to ~27% of the wild type infection level (Fig. 7D, compare bar 4 to 1). Whereas, the β-Gal positive cell levels for sc-virus infection in siRNA-treated cells and for siRNA virus infection in the control cells were slightly decreased to 76 and 70% of the wt infection levels (Fig. 7D, compare bars 2 and 3 to bar 1). All of these results indicate that Imp7 knockdown in both HIV-1 producing and target cells impairs HIV-1 infection.

**DISCUSSION**

HIV-1 IN is a key enzymatic molecule that has been shown to contribute to different steps during the early stage of HIV-1 replication, including reverse transcription, viral DNA nuclear import, and integration. Even though the exact mechanisms underlying the action of IN during each of these critical early steps is not fully understood, accumulative evidence indicates that IN is capable of interacting with different viral and cellular proteins at various steps during HIV-1 replication. This viral protein is well documented to possess karyophilic properties, and mutagenic analysis has revealed that some IN mutants significantly affect HIV-1 nuclear import (7, 10, 31–34, 50). Several studies have showed that IN is capable of binding to Impα and/or Imp7 in *in vitro* binding assays, suggesting that HIV-1 IN may recruit these cellular nuclear import factors during HIV-1 nuclear import (7, 36, 37). However, whether these cellular factors contribute to HIV-1 DNA nuclear import and replication still remains controversial (33, 37, 38, 44). In this study, we have used a cell-based co-IP approach to investigate the interaction occurring between HIV-1 IN and several cellular nuclear import factors. Our results clearly show that HIV-1 IN, in both IN-YFP and YFP-IN fusion protein forms, specifically interacts with Imp7, but is unable to bind to Imp8 and Impα (Rch1). This specific IN/Imp7 interaction was further confirmed by using tandem affinity purification-tagged IN (TAP-IN) (data not shown). We further tested the interaction of HIV-1 IN with endogenous Imp7, our results demonstrated that the endogenous Imp7 was co-precipitated with IN-YFP in 293T cells (Fig. 2B), or co-pulled down with IN-HA in HIV-1-infected CD4+ T cells (Fig. 2C). Furthermore, our *in vitro* binding experiments revealed that the purified GST-Imp7 was able to pull down purified recombinant HIV-1 IN in both dimer and monomer forms. Thus, all of these studies provide evidence that HIV-1 IN specifically interacts with Imp7.

Another HIV-1 karyophilic protein MAP17 was also implicated in HIV-1 nuclear import. However, unlike IN, which was shown to be required for HIV-1 nuclear import in both dividing and non-dividing cells, MAP17 contributes to HIV-1 nuclear import mainly in non-dividing cells (see reviews in Refs. 2 and 51). In this study, we have compared the binding ability of these two HIV-1 proteins to Imp7 and Impα (Rch1). Interestingly, in contrast to IN, the MAP17 is unable to interact with Imp7 (Fig. 3). On the other hand, whereas IN fails to bind to Impα (Rch1), MAP17 is shown to interact with Impα, which confirmed the previous observation by Gallay et al. (6) showing that HIV-1 MAP17 binds to Rch1 in a co-IP experimental approach. These observations suggest that HIV-1 IN and MAP17 may interact with different cellular machineries during HIV nuclear import, and/or replication. How these viral/cellular protein interactions synergize to assist with HIV-1 replication, especially in non-dividing cells, remains an interesting question to be addressed.

Our deletion analysis identified that the Imp7-binding site(s) lies in the C-terminal domain of IN. The function of the C-terminal domain of IN was originally ascribed to that of nonspecific DNA binding, leading to a suggestion that this domain may contribute to chromosomal DNA recognition during viral DNA integration (52–54). In addition, several recent studies indicate that the C-terminal domain of IN contribute to multiple steps during the early stage of HIV-1 replication, including reverse transcription, nuclear import, and/or the postnuclear entry step(s) (30, 32, 34, 55, 56). In this study, two regions, 235WWPGPKLLWWKG and 262RRKAK, within the C-terminal domain of IN were identified to contribute to the IN/Imp7 interaction. To investigate the effect of the IN/Imp7 interaction on HIV-1 replication, a VSV-G pseudotyped HIV-1 virus (vKKRK) containing the Imp7-binding defect IN mutant was produced. Infection analysis revealed that the vKKRK virus induced even lower luciferase activity than that of the integration-defective class I mutant D64E virus, indicating that this virus is replication defective. Further analysis showed that the Imp7-binding defective virus displayed impairments at both viral reverse transcription and nuclear import (Fig. 6, C and D). Because this virus was shown to be non-infectious in C8166 cells (Fig. 6B), it is expected that this mutant virus also affects virus integration. Consistently, previous studies have already shown that several IN mutants targeting these positively charged residues inhibited HIV-1 integration (32, 55). Given that most IN class II mutants cause pleiotropic damage during viral replication (55–57), we could not conclude that these defects solely resulted from the lose of the IN/Imp7 interaction. However, it is conceivable that IN/Imp7 interaction may have contributed to these critical steps during HIV-1 replication.

Another approach to validate the functional role of the IN/Imp7 interaction in HIV-1 replication is to directly target Imp7 expression within susceptible cells. Fassati et al. (38) previously showed that siRNA-mediated knockdown of endogenous Imp7 inhibited HIV infection. However, a recent study by Zieleske and Stevenson (44) did not reveal an inhibitory effect of Imp7 knockdown on HIV-1 nuclear import. It is worth noting that these studies were mainly focused on wild type HIV-1 infection in Imp7 knockdown-susceptible cells. It could not rule out the possibility that Imp7 might have an effect on late stage of virus replication, and/or be packaged into viral particles and playing a role in subsequent viral infection. Indeed, the study by Zieleske and Stevenson (44) observed a slight decrease of 2 long terminal repeat formations in Imp7-depleted target cells, infected with viruses produced from a single dose siImp7-treated cells, in which Imp7 mRNA levels was reduced to 77% at the time of virus collection. In this study, we have compared infections in siRNA- or scRNA-depleted cells with viruses that were produced from either siRNA- or scRNA-depleted 293T cells. Interestingly, results showed that depletion of Imp7 in
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both HIV-1 producing and target cells lead to a 2.5–3.5-fold decrease of HIV-1 infection, as measured by either HIV-1-induced luciferase activity or the amount of β-Gal positive cells (Fig. 7, C and D). However, such reduced HIV-1 replication was not observed, for the infection of Imp7-depleted cells with normal virus, or the infection of normal cells with viruses produced from Imp7-depleted cells. Even though the mechanism underlying the Imp7 knockdown-mediated inhibition of HIV-1 replication is still undefined, these results indicate that Imp7 contributes to efficient HIV-1 replication.

It should also be noted that the Imp7 knockdown in the producer-target cell combination system only induced 2.5–3.5-fold reduction of viral infection. This result leads us to consider several possibilities. It is possible that HIV-1 IN may have the ability to interact with multiple cellular nuclear import factors, and dissociation of one of them is not sufficient to abolish HIV-1 replication. Similarly, it was shown that both the cellular ribosomal protein and the glucocorticoid receptor, utilize Imp7 and Impo/Impβ as its nuclear import receptors (18, 26). Another possibility could be that IN interacts with a nuclear import receptor complex, in which Imp7 may act as an accessory cofactor. Indeed, previous studies have demonstrated that Imp7 is capable of forming a heterodimer with Impβ, and this heterodimeric complex has a higher binding affinity for histone H1 (25, 27). Interestingly, it was also shown that the Impβ/RanGTP interaction appears to be essential for histone H1 import, whereas Ran binding of Imp7 is dispensable (27). In addition, the limitation of siRNA knockdown technology used for this particular study should also be under consideration, because Imp7-siRNA treatment could not erase residual levels of Imp7 from cells, and such low amounts of Imp7 may still be recruited by IN to support a lower level HIV-1 infection. Therefore, a genetic knock-out cell line will be required to address the impact of Imp7 on HIV-1 infection, as is being carried out to fully understand how this cellular nuclear import receptor contributes to efficient HIV-1 replication.

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