The HIV-1 Integrase Mutant R263A/K264A Is 2-fold Defective for TRN-SR2 Binding and Viral Nuclear Import*

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Background: Whether the TRN-SR2/HIV-1 IN interaction mediates the nuclear import of HIV remains controversial.

Results: The replication-defective HIV integrase mutant INR263A/K264A displays a 2-fold reduction in interaction with TRN-SR2 and PIC nuclear import.

Conclusion: The HIV-IN TRN-SR2 protein-protein interaction is important for HIV nuclear import.

Significance: The IN/TRN-SR2 interaction interface is a potential target for future antiviral therapy.

Transportin-SR2 (Tnpo3, TRN-SR2), a human karyopherin encoded by the TNPO3 gene, has been identified as a cellular cofactor of HIV-1 replication, specifically interacting with HIV-1 integrase (IN). Whether this interaction mediates the nuclear import of HIV remains controversial. We previously characterized the TRN-SR2 binding interface in IN and introduced mutations at these positions to corroborate the biological relevance of the interaction. The pleiotropic nature of IN mutations complicated the interpretation. Indeed, all previously tested IN interaction mutants also affected RT. Here we report on a virus with a pair of IN mutations, INR263A/K264A, that significantly reduce interaction with TRN-SR2. The virus retains wild-type reverse transcription activity but displays a block in nuclear import and integration, as measured by quantitative PCR. The defect in integration of this mutant resulted in a smaller increase in the number of two-long terminal repeat circles than for virus specifically blocked at integration by raltegravir or catalytic site mutations (IND64N/D116N/E152Q). Finally, the replication-defective HIV integrase mutant INR263A/K264A displays a 2-fold reduction in interaction with TRN-SR2 and PIC nuclear import.

The replication-defective HIV integrase mutant INR263A/K264A displays a 2-fold reduction in interaction with TRN-SR2 and PIC nuclear import. These data altogether underscore the importance of the HIV-IN TRN-SR2 protein-protein interaction for HIV nuclear import and validate the IN/TRN-SR2 interaction interface as a promising target for future antiviral therapy.

Lentiviruses such as the HIV-1 infect both dividing and non-dividing cells (1, 2). Following reverse transcription, the preintegration complex (PIC) composed of the viral DNA and a complex of viral and cellular proteins crosses the nuclear mem-

brane via active nuclear import through the nucleopore (3). Nuclear entry precedes provirus establishment through stable insertion of the viral DNA into the host chromatin as catalyzed by HIV-1 integrase (IN) and constitutes a genuine bottleneck during HIV replication (4). As such, it represents an interesting, yet unexploited target for antiviral therapy. Despite extensive research, the underlying mechanism of HIV nuclear import remains a contentious issue until today.

Several viral components, including capsid (CA), the central polypurine tract, IN, matrix, and Vpr (viral protein R) have been reported to play a role in nuclear import, but no consensus has been reached (5–7). Nuclear import in the human cell is orchestrated by a plethora of karyopherins, comprising both broad spectrum and highly specialized transporters. Several of these have been suggested to take part in HIV nuclear import, in particular importin-α/β (8–10), importin-α3 (11), and importin-7 (12–15). In 2008, transportin-SR2 (TRN-SR2, transportin-3, Tnpo3), encoded by the TNPO3 gene, was independently identified as a cellular cofactor of HIV in two genome-wide siRNA screens (16, 17) and picked up as a specific binding partner of HIV IN (18). Using RNAi technology, the crucial role of TRN-SR2 in HIV replication was demonstrated (16–18).

Although the importance of TRN-SR2 for HIV replication is widely accepted and has been independently confirmed by different research groups, the exact mechanism of action remains unresolved (19–29). Because TRN-SR2 is a known karyopherin, involved in nuclear import of essential splicing factors, namely serine/arginine-rich proteins and also certain non-serine/arginine-rich proteins (30–33), it is a plausible candidate import factor for HIV.

IN catalyzes the insertion of the viral DNA into the host chromatin in two distinct steps. During 3′-processing, the enzyme removes the 3′-terminal GT dinucleotide from both long terminal repeat (LTR) ends, resulting in reactive 3′-OH groups. This reaction is believed to occur in the cytoplasm. The processed cDNA is then imported in the nucleus where a nucleophile attack of the exposed 3′-OH groups on the target DNA phosphodiester backbone and transesterification result in stable insertion of the viral DNA into the host chromatin. During HIV infection, a minute fraction of the proviral cDNA is
subjected to one- and two-LTR circle formation in the nucleus, which can be monitored by Q-PCR (34). IN catalytic site mutations or the addition of integrase inhibitors during HIV infection reproducibly result in abortive integration and a steep increase in two-LTR circles (34), whereas a decrease in two-LTR circles is generally accepted to reflect a block in nuclear import (35). In line, a reduction in the number of two-LTR circles was measured after RNAi-mediated TRN-SR2 depletion (18, 20, 21, 36), suggesting a nuclear import defect. In a direct HIV nuclear import assay using IN-eGFP-labeled PICs (18, 37), we demonstrated a reduction of nuclear versus cytoplasmic PICs after TRN-SR2 knockdown (18), underscoring a role for TRN-SR2 in HIV nuclear import.

Although we and others have shown that TRN-SR2 directly interacts with HIV IN (18, 19, 27, 38, 39), the mechanism of action has been questioned (19, 23, 36, 41). Especially reports on a set of HIV capsid mutations (e.g. N74D) that reduce the dependence of HIV replication on TRN-SR2 in single-round HIV infection assays put forward an alternative hypothesis according to which TRN-SR2 exerts its role through direct interaction with the viral CA protein (19, 23). However, in multiple-round, spreading infection experiments, HIV carrying the N74D CA mutation remained sensitive to TRN-SR2 depletion (25). Possibly, these capsid mutations affect virus uncoating (25, 42) and thus constitute a rate-limiting step prior to HIV nuclear import. Initially, the N74D capsid mutant was picked up as a resistance mutation against overexpression of mCPSF6–358, a C-terminally truncated fragment of mCPSF6 (mouse cleavage and polyadenylation specificity factor subunit 6) (43). CPSF6 is a cellular protein involved in splicing and polyadenylation of pre-mRNA that carries an R5 domain at its C terminus. Because CPSF6 binds HIV CA (40), it has been suggested that TRN-SR2 depletion might result in cytoplasmic accumulation of CPSF6, which in turn might perturb viral uncoating through CPSF6-CA binding, leading to an indirect block in nuclear import and restricted HIV replication (36, 40, 41). Still, the cytoplasmic accumulation of CPSF6 after TRN-SR2 knockdown is not always observed (36, 41). In any case, the fact that a virus defective for interaction with CPSF6 (CA N74D) remains sensitive to TRN-SR2 knockdown in a multiple-round replication experiment (25) suggests that cytoplasmic accumulation of CPSF6 is not the sole cause for the HIV replication deficit upon TRN-SR2 depletion.

These indirect lines of evidence do not unequivocally address the question whether the IN/TRN-SR2 interaction mediates nuclear entry. To study its role in HIV nuclear import, a specific block in the IN/TRN-SR2 interaction is required. To uncouple the effect of TRN-SR2 knockdown from the possible cytoplasmic accumulation of CPSF6 or other cargoes, we decided to search for IN mutants specifically defective for TRN-SR2 binding. We previously characterized the TRN-SR2 interaction interface to identify key amino acids in the viral IN and determined the hot spots for the interaction, namely the amino acids Arg262-Arg263-Lys264 and Lys266-Arg269 in IN (27). Independent confirmation of the role of Arg262, Arg263, and Lys264 was provided by Larue et al. (38).

We next decided to introduce specific IN interface mutants in NL4-3 backbones to study their effect on viral replication. However, because IN is an essential component of the nucleo-protein complex in the budding virus and of the reverse transcription complex, IN mutations can result in pleiotropic effects extending well beyond the integration step. It is well established that some IN mutants negatively affect reverse transcription (44, 45). Of the five amino acids identified as hot spots for interaction with TRN-SR2 (see Fig. 1), the K266A and R269A single point mutants, as well as the double mutants R262A/R263A and R262A/K264A, were reported before to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as blocked nuclear import.

Here we describe a virus carrying an IN mutant with reduced affinity for TRN-SR2 (NL4-3 IN R266A/K264A) that still displays wild-type reverse transcriptase activity. Demonstration of a nuclear import defect for this virus implies that the interaction between IN and TRN-SR2 mediates HIV PIC nuclear import.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Recombinant Protein Purification—**Recombinant proteins were expressed in *Escherichia coli* strain BL21-CodonPlus (DE3). Recombinant His<sub>6</sub>-tagged HIV-1 IN (46) and recombinant GST-tagged TRN-SR2 (18) were purified as described previously. His<sub>6</sub>-tagged HIV-1 IN was expressed using pLNSD.His (obtained through the AIDS Research and Reference Reagent Program). The mutations in pLNSD.His were introduced by site-directed mutagenesis using the Kirsch and Joly method (47). Megaprimers were ordered and used to perform a full round PCR on the pLNSD.His plasmid. For the IN<sup>N74D</sup> mutation, the following megaprimers were used: pLNSD-RK263toA-s: 5′-CAATCATCAGGCTGATCTGGTTCTCCATAAATCCCTGGATCTTGTGCTGGCTTGGGACTACTTTATTTCACTATATTTCTGG-3′ and pLNSD-RK263toA-as: 5′-CGAGATTAATGGCATATAAAATGGATTGCGCAAGACAGCCAGGTGATGATTG-3′. All plasmid constructs used in this work were verified via sequence analysis.

**AlphaScreen Protein-Protein Interaction Assay—**The AlphaScreen binding assay was performed as described previously (27). In brief, proteins were all diluted to 5 × working solutions in the assay buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2 mM DTT, 0.1% (v/v) Tween 20, and 0.1% (w/v) BSA). First, 10 μl of the GST-TRN-SR2 was pipetted into the wells, followed by 5 μl of a His<sub>6</sub>-IN dilution series. The plate was sealed and left to incubate for 1 h at 4°C. Next, 10 μl of a mix of Ni<sup>2+</sup> chelate acceptor and glutathione donor AlphaScreen beads (PerkinElmer Life Sciences) was added. The plates were then incubated for 1 h at 30°C and analyzed using an EnVision multilabel reader (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Points for which quenching of the signal occurred through excess of either binding partner (“hooking” as referred to by the supplier) were excluded from the final plot.

**Integrate Activity Assays—**3′-Processing activity was measured by detection of radioactively labeled products by denaturing gel electrophoresis and was performed as described previously (48). Briefly, 500 nM IN was incubated for 7.5 min at 37°C with 20 nM of the radioactively labeled oligonucleotide substrate (INT1, 32P-5′-GTGTTGAAATCTCCTAGCAGT-
Cell Culture—HeLaP4 cells (obtained from the National Institutes of Health Reagent Program) were grown in DMEM supplemented with 50 μg/ml gentamicin (Invitrogen) and 5% fetal calf serum (International Medical). The cells were incubated at 37 °C and 5% CO2 in a humidified atmosphere. Two different HeLaP4 cell lines stably knocked down for importin-α3 were generated by transduction with two different lentiviral vectors expressing shRNAs targeting the importin-α3 mRNA, called sh93 and sh97 (Sigma; clones NM_002268.3–1814s1c1 and NM_002268.3–605s1c1). A control cell line was established using a control vector expressing a scrambled shRNA referred to as shSCR (Sigma; product number SHC002). These lentiviral transfer plasmids were a kind gift from Dr. R. Hoeben (Leiden University Medical Center, Leiden, The Netherlands). The shRNA expressing transfer plasmids are based on the pLKO.1 plasmid (Sigma) containing a puromycin resistance cassette. After transduction, cells were selected and grown in DMEM as described above with the addition of 1 μg/ml puromycin. MT-4 cells (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, from Dr. Douglas Richman) were grown in RPMI 1640 (Invitrogen 461 BRL) supplemented with 12% FCS and 50 μg/ml gentamicin. Human peripheral PBMCs were purified from fresh buffy coats of anonymous voluntary donors using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) following the manufacturer’s protocol. Subsequently, PBMCs were maintained and stimulated in RPMI 1640 supplemented with 15% FCS and 20 units/ml IL-2 and IL-10.

Western Blotting—For Western blotting, protein concentrations of 1% SDS whole cell extracts were determined using the BCA protein assay (Thermo Scientific Pierce), and after separation by SDS-PAGE, 25 μg of each extract was electrophoresed onto polyvinylidene difluoride membranes. Membranes were probed with monoclonal antibodies against importin-α3 (1:500 dilution) (ab6039; Abcam), and β-actin (1:5000 dilution) (A5441; Sigma) antibodies were used to confirm equal loading. Detection was performed using horseradish peroxidase-conjugated antibodies (Dako) and chemiluminescence (Pierce ECL). The extent of 3′-processing was measured based on the respective intensity of 3′ bands relative to the total radioactivity present in the lane. These data were determined using the OptiQuant Acquisition and Analysis software (PerkinElmer Life Sciences). Strand transfer activity was measured with a quantitative ELISA as described previously (49).

For the quantification of two-LTR circles, the forward primer 3′-GCTATGTC-3′ (Invitrogen). This plasmid was then used to perform Kirsch and Joly site-directed mutagenesis using the pNNSD-RK263toA-s and pNNSD-RK263toA-as primers. The mutated IN sequence was then cloned back into pNL4-3 or pNL4-3.Luc.RE—using two unique restriction sites (Agel and PflMI). Viruses were produced by polyethyleneimine-mediated transfection of 293T cells using 20 μg of the viral molecular clone pNL4-3 per 10-cm dish, for vectors an extra 5 μg of the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein (50) was added. 2 and 3 days post-transfection, the supernatant was harvested, filtered through 0.22-μm pore-size syringe filters (Sartorius), concentrated by centrifugal filtration using Vivasin concentrators (Millipore), and treated with DNase I (Roche Applied Science) for 1 h at 37 °C. For the single round experiments, HeLaP4 cells (106 cells) stably depleted of importin-α3 and control cells were seeded into 96-well plates. One day later cells were infected in triplicate with two dilutions (21,000 and 7,000 pg of p24) of vesicular stomatitis virus glycoprotein pseudotyped single-round HIV-1 virus in a total volume of 100 μl per well. Each well was lysed 2h after infection using 50 μl of lysis buffer (50 mM Tris/HCl, pH 7.3, 200 mM NaCl, 0.2% Nonidet P-40, 5% glycerol) and was analyzed by firefly luciferase activity (ONEGlo™; Promega) according to the manufacturer’s protocols. Chemiluminescence was measured with a Glomax luminometer (Promega). The protein concentration of each sample was determined (BCA protein assay kit; Thermo Scientific Pierce), and readouts were normalized to 1 μg of total protein. For the breakthrough experiments, HeLaP4 cells (3.10⁵), MT-4 cells (10⁶ cells) or PBMCs (10⁶ cells) were infected with virus amounting to 30,000, 50, or 20,000 pg of p24, respectively. Starting 3 days after infection, supernatants were sampled daily.

Q-PCR Quantification of HIV DNA Species—10⁶ MT-4 cells were infected with 10⁶ pg of p24 of virus. Inhibitors, if present, were 2 μM zidovudine or 0.6 μM raltegravir (Ral). At 2h post-infection, supernatants were replaced with fresh DMEM containing DMSO, zidovudine, or Ral. Ritonavir (4 μM) was added to all samples to restrict replication to a single round. At distinct time points after infection, cells were lysed by the addition of a 10 mM Tris (pH 8.0) buffer containing 0.02% Triton X-100, 0.02% SDS, 1 mM EDTA, and 0.8 mg of proteinase K/ml and incubated for 1 h at 56 °C. Q-PCR was performed directly on the lysates. A specifically designed set of TaqMan probe and primers was used to quantify the amount of each specific HIV-1 DNA form in the cell lysate as described previously (34, 51, 52). For the quantification of reverse transcripts (total HIV-1 DNA), the forward primer 5′-ATCAAGCAGCCATGCAAATGTT-3′, the reverse primer 5′-CTGAGGGGTACTAGTGTGTCATGTC-3′, and the probe 5′-(FAM)-TCCACACTGACTAAAAGGGTCTGAGG-3′ were used. For the quantification of two-LTR circles, the forward primer 5′-GTGCCCGTCTGTGGTGTGACT-3′, the reverse primer 5′-CTTGTCTTCTTTGGGAGAGAATTAGC-3′, and the probe 5′-(FAM)-ACCATCAAT-GAGGAAGTCGAGAACATGGGA-(TAMRA)-3′ were used. For the quantification of two-LTR circles, the forward primer 5′-GAGAACAGTCTATGGAGGCTGGTTGATC-3′, the reverse primer 5′-TCCACACTGACTAAAGGGTCTGGAGGTCTCT-(TAMRA)-3′ were used. Quantification of integrated proviruses was performed by nested Alu-PCR, as described previously (51). For the first round, the forward primer 5′-CTAACATAGGAACCCCAGCTGTA3′ and the reverse primer 5′-TGCTGGATTACAGGCCTGAG-3′ were
used. The reaction contained 2 mM MgCl$_2$, 250 μM dNTPs, 400 nM primers, 1.25 units of Taq DNA polymerase (Fermentas), 75 mM Tris-HCl (pH 8.8), 20 mM (NH$_4$)$_2$SO$_4$, and 0.01% Tween 20. For the Q-PCR, the forward primer 5′-AGCGTGCCCTGAAGTGGT-GCTTCAA-3′, the reverse primer 5′-TGAAGAAAGGTTCTGAGGGATCT-3′, and the probe 5′-(FAM)- TTACCA-GAGTGACACACACAGCGGGA-(TAMRA)-3′ were used. In each sample RNase P DNA was quantified as the endogenous control (Applied Biosystems). For each real time PCR analysis, a standard curve was generated using dilutions of a representative sample. No-template controls (no DNA added to the PCR mixture) were run with each experiment. Reactions contained 1× iTaq supermix (Bio-Rad), 300 nM forward and reverse primers, and 200 nM probe. After initial incubations at 95°C for 5 min, 50 cycles of amplification were carried out at 95°C for 10 s, followed by 30 s at 55°C. The reactions were analyzed using the LightCycler® 480 (Roche Applied Science).

**PIC Nuclear Import Assay**—The PIC nuclear import assay was performed with adaptations to the previously published protocol (37). 293T cells were transfected with 15 μg of pVpr-eGFP (Vpr and IN were cloned into the pEGFP-N1 vector from Clontech), 15 μg of pNL4-3.Luc.R (obtained from the AIDS Reference and Reagent Program), and 5 μg of the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein (50). Supernatant was collected after 48 h, filtered through a 0.45-μm pore size filter (Sartorius), and then concentrated by ultracentrifugation at 27,500 rpm for 1 h 45 min. p24 was measured with the INNOTEST HIV antigen mAb kit of Innogenetics. A viral inoculum of 3.10$^6$ pg of p24 was used to infect 30,000 HeLa-P4 cells. Five hours after infection, cells were fixed with 2% paraformaldehyde. The nuclear lamina was visualized by staining with a monoclonal anti-lamin A/C antibody (Santa Cruz; sc-7292) and a secondary goat anti-mouse IgG Alexa-Fluor 633. Three-dimensional stacks of fixed cells were acquired with a Zeiss LSM510 multiphoton confocal microscope (Cell Imaging Core CIIC, University of Leuven) equipped with a Plan-Apochromat 63×/1.4 oil DIC (differential interference contrast) objective. The z-step size was 0.3 μm. Ar and HeNe laser lines were used for EGFP and Alexa 633 excitation. A sequential image acquisition was used. The quantification of the PICs was performed using a homemade MatLab routine (The MathWorks, Inc.). In brief, after adequate image processing, points were automatically detected using an intensity threshold. A fluorescent spot was assigned as a PIC if at least two connecting pixels are above the threshold and if the fluorescent signal is present in at least two consecutive frames (z planes). PICs were classified as cytoplasmic, in the nucleus or at the nuclear membrane based on the nuclear lamina staining.

**RESULTS**

**Selection of Mutants Defective for IN/TRN-SR2 Interaction to Study Nuclear Import of HIV**—We previously characterized the TRN-SR2 interaction interface to identify key amino acids in viral IN. Next, we introduced these specific IN interface mutants in NL4-3 backbones to study their effect on viral replication. Of the five amino acids identified as hot spots for interaction with TRN-SR2 (Fig. 1), the K266A and R269A single point mutants, as well as the double mutants R262A/R263A and R262A/K264A, were reported before to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as a possible block in nuclear import. Therefore we tested the remaining mutants, namely the single point mutants R262A, R263A, K264A, and the double point mutant R263A/K264A for their binding to TRN-SR2 using AlphaScreen (Fig. 1B). AlphaScreen is a bead-based protein-protein interaction assay in which binding of the proteins results in light emission (AlphaScreen signal). The single point mutants R262A, R263A, and K264A did not affect TRN-SR2 binding (data not shown), whereas INR263A/K264A showed a reduction in AlphaScreen signal. We therefore performed an outcompetition AlphaScreen experiment to quantify the change in affinity to TRN-SR2 compared with WT IN. We outcompeted the binding between GST-TRN-SR2 and His-tagged WT IN or INR263A/K264A with untagged CTD of IN and calculated the IC$_{50}$ of IN CTD in three independent experiments each performed in duplicate. The IC$_{50}$ for outcompetition with IN CTD was 172 nM (95% confidence interval (CI) = 24.6–237.9 nm) for WT IN and 74 nm (95% CI = 45.95–119.5 nm) for INR263A/K264A, pointing to a significant 2.3-fold lower affinity of mutant IN for TRN-SR2 than WT IN (p = 0.0154; Fig. 1B).

Next we compared the catalytic activities of WT IN, the double mutant INR263A/K264A and the enzymatically dead IND64A (53). The 3′-processing activity was severely affected, because INR263A/K264A showed only residual activity in comparison with WT IN (Fig. 1C). Both INR263A/K264A and IND64A were defective for strand transfer as determined in a quantitative ELISA assay (Fig. 1D). The lack of catalytic activity of the INR263A/K264A mutant, however, did not hamper the analysis of reverse transcription or nuclear import because these steps precede integration. In a previous study, we showed using size exclusion chromatography that the R262A/R263A/K264A substitutions in IN do not affect IN oligomerization (27). We hence decided to study INR263A/K264A in the context of the virus.

**Characterization of HIV-1 NL4-3 Virus Carrying INR263A/K264A**—The INR263A/K264A mutations were inserted into a molecular clone of HIV (pNL4-3). Mutant and WT virus were harvested after transient transfection of 293T cells and normalized for p24 level or reverse transcriptase activity. HeLaP4, MT-4, and PBMCs isolated from donor blood samples were infected with equal amounts of WT or INR263A/K264A virus. Viral replication was monitored by sampling the supernatant as long as cells were viable. As expected from the reduced IN activities, INR263A/K264A virus was completely replication-deficient in primary cells (Fig. 1E), as well as in HeLaP4 and MT-4 cells (data not shown).

In 2010, a potential role of importin-α3 in HIV nuclear import was reported (11). Later the IN amino acids Arg$^{63}$ and Lys$^{264}$ were claimed to be involved in the binding to importin-α3 (54). We therefore analyzed the effect of importin-α3 knockdown on HIV replication. We generated two stable importin-α3 knockdown cell lines (sh93 and sh97) and infected those with HIV NL4.3-Fluc. Potent knockdown of importin-α3 (95 and 99%, respectively) did not significantly reduce HIV infection compared with control cells (shSCR) (Fig. 2). In comparison, a 70% knockdown of TRN-SR2 resulted in a 10-fold
reduction of HIV infection (18). Because of the uncertain role for importin-α3 in HIV-1 infection, the INR263A/K264A mutant was used to study the specific role of the IN/TRN-SR2 interaction during HIV nuclear import.

To pinpoint at which step viral replication of the INR263A/K264A virus was blocked, we monitored formation of viral DNA intermediates during single-round HIV-1 infection by Q-PCR for late reverse transcripts, integrated copies, and two-LTR circles.
To control for the 3’-processing deficiency of IN<sup>R263A/K264A</sup>, we compared the mutant not only to WT virus but also to the NL4-3 IN<sup>D64N/D116N/E152Q</sup> mutant virus (55). We generated this triple mutant, which has a mutated catalytic triad and lacks all enzymatic activity, as a more stringent control than the D64A mutant used previously (53).

Late reverse transcripts were measured by Q-PCR at 4, 8, 10, and 24 h postinfection (Fig. 3, A and B). The HIV NL4-3 IN<sup>R263A/K264A</sup> mutant produced a similar number of reverse transcripts (taken at 8 and 10 h postinfection) as WT virus, whereas the IN<sup>D64N/D116N/E152Q</sup> mutant produced less reverse transcripts (93.6 ± 8.8% for NL4-3 IN<sup>R263A/K264A</sup> p = 0.369 and 68.9 ± 4.5% for NL4-3 IN<sup>D64N/D116N/E152Q</sup> p = 0.001). Conversely, the chain terminator zidovudine effectively blocked reverse transcription of all viruses, whereas the strand transfer inhibitor Ral did not affect reverse transcription (Fig. 3, A and B). Next, integrated viral DNA was measured by Q-PCR at 48 h postinfection. The number of integrated copies of WT NL4-3 was effectively reduced by the addition of zidovudine or Ral. No further increase upon addition of Ral was observed as expected for a catalytically dead mutant (Fig. 3F). The Q-PCR data on the two-LTR circles of the IN<sup>R263A/K264A</sup> mutant require careful analysis because a block of integration will typically result in increased levels of two-LTR circles, but a concomitant block in nuclear import will lead to reduced two-LTR circle formation (18, 20, 21, 36). Although the number of two-LTR circles of the IN<sup>R263A/K264A</sup> mutant was 2-fold higher than that of WT virus (Fig. 3E), this increase was much lower than expected for a virus affected at the integration step (on average 10-fold higher) (Fig. 3F). Even though the different viral preps were normalized for p24 and showed a comparable extent of reverse transcription, two-LTR circles of the IN<sup>R263A/K264A</sup> mutant increased to levels that were 2.5-fold lower (p = 0.0004) than those of the catalytic dead mutant. Upon addition of Ral to WT virus, a 10-fold increase in two-LTR circles was measured (p = 0.0002), which is typically seen with a block in integration. These data indicate that NL4-3 IN<sup>R263A/K264A</sup> is also affected at a step following reverse transcription and before integration, thus most presumably at the nuclear import step.

**Nuclear Import Defect of NL4-3 IN<sup>R263A/K264A</sup> Virus—To confirm the effect of IN<sup>R263A/K264A</sup> on HIV nuclear import, we finally performed a PIC nuclear import assay (37) (see “Experimental Procedures” for more details). We produced two distinct IN-eGFP-labeled viruses: one with WT IN and the other with the IN<sup>R263A/K264A</sup> double mutant. Five hours after infection, the fluorescently labeled PICs were visualized using confocal microscopy (Fig. 4A). The ratio of nuclear PICs over cytoplasmic PICs (percentage of nuclear PICs) is a measure of nuclear import (18). To analyze HIV carrying IN<sup>R263A/K264A</sup>, two independent experiments, with distinct productions of both the WT and the IN<sup>R263A/K264A</sup> mutant virus, were performed. Cytoplasmic and nuclear PICs were counted (Fig. 4B). The ratio of nuclear versus cytoplasmic PICs was calculated for both independent experiments. The difference between the distributions of the percentage of nuclear PICs for WT and IN<sup>R263A/K264A</sup> mutant PICs was highly significant (WT, n = 51 cells; IN<sup>R263A/K264A</sup>, n = 49 cells; p = 0.0054, Mann-Whitney test; and WT, n = 41 cells; IN<sup>R263A/K264A</sup>, n = 44 cells; p = 0.0095, Mann-Whitney test) (Fig. 4B). Combining the data sets of the two individual experiments the p value further decreased to 0.0001 (WT, n = 92 cells; IN<sup>R263A/K264A</sup>, n = 93 cells). The mean percentage of nuclear PICs calculated with 95% confidence interval was 4.1% for the WT (95% CI, 3.5%, 4.7%) and 2.4% for IN<sup>R263A/K264A</sup> (95% CI, 2.1%, 2.8%). When the percentage of cells that maximally contain a specific number of nuclear PICs was plotted, 50% of the IN<sup>R263A/K264A</sup> mutant virus-infected cells contained 0–1% nuclear PICs, whereas 50% of the WT virus-infected cells contained 0–3% nuclear PICs, in line with a reduced nuclear import for HIV IN<sup>R263A/K264A</sup> and corroborating the Q-PCR results.

In a following experiment, we evaluated the combined effect of TRN-SR2 depletion and interface mutants on PIC nuclear import. Stable TRN-SR2 depletion (shTR3) resulted in a signif-
significant reduction in nuclear PICs compared with control cells (shSCR) (WT in shSCR cells, n = 55 cells; WT in shTR3 cells, n = 49 cells; p = 0.0023, Mann-Whitney test) (Fig. 4C) as shown previously after transient depletion of TRN-SR2 (18). The mean percentage of nuclear PICs calculated with 95% confidence intervals was 2.2% for the control cells (95% CI, 0.8%, 3.6%) and 0.5% for TRN-SR2 knockdown cells (95% CI, 0.3%, 0.7%). We plotted the percentage of cells that maximally contain a specific percentage of nuclear PICs. For example, 95% of the TRN-SR2 knockdown cells contain 0–2% nuclear PICs, whereas 95% of the control cells contain 0–5% nuclear PICs. In parallel the INR263A/K264A virus was tested both in shSCR cells (n = 50 cells) and in shTR3 cells (n = 50 cells). Also in this experiment, nuclear import of the WT virus was significantly higher than that of the INR263A/K264A mutant virus; p = 0.0167. In shTR3 cells, data for the WT virus (mean percentage of nuclear PICs = 0.5) were not statistically different from data for the INR263A/K264A mutant virus in shSCR cells (mean percentage of nuclear PICs = 0.7; p = 0.5836). For the INR263A/K264A mutant virus in shTR3 cells, the mean percentage of nuclear PICs was calculated with 95% confidence intervals as 0.2% (95% CI, 0.1%, 0.3%), which was significantly different from the INR263A/K264A mutant virus in shSCR cells (p = 0.002), indicating an additive effect of TRN-SR2 depletion and interface mutations.

DISCUSSION

In 2008, we identified TRN-SR2 as an interaction partner of HIV-1 integrase by yeast two-hybrid screening (18). Using RNAi technology, we could demonstrate the crucial role of TRN-SR2 during HIV replication both in dividing and nondividing cells.
viding cells (18). Independently, two genome-wide siRNA screens also identified TRN-SR2 as a crucial cofactor for HIV replication (16, 17). While this paper was in revision, two papers were published revealing the crystal structure of TRN-SR2 (57, 58). The TRN-SR2 mutant, D750R/D751R, was defective for interaction with CPSF6 and proved unable to support HIV-1 infection although reportedly retaining WT level of HIV IN and ASF/SF2 binding (57). In our hands, TRN-SR2D750R/D751R displayed a slightly reduced affinity for IN but was strongly impaired for binding to unphosphorylated ASF/SF2, suggesting that this mutant is generally reduced in its cargo binding capacity (data not shown).

Although indirect evidence suggests that the IN/TRN-SR2 interaction mediates HIV nuclear import, we thus needed to specifically block this interaction to corroborate its role in HIV nuclear import. To uncouple the effect of TRN-SR2 knockdown from the possible cytoplasmic accumulation of CPSF6 or other cargoes, we decided to search for IN mutants selectively defective for TRN-SR2 binding. We previously characterized the TRN-SR2 interaction interface to identify key amino acids in IN and determined as hot spots the amino acids Arg262-Arg263-Lys264 and Lys266-Arg269 (27). Independent confirmation of the role of Arg262, Arg263, and Lys264 was provided by Larue et al. (38).

Here we aimed to insert specific IN interface mutants in HIV molecular clones to study their effect on viral replication. However, of the five amino acids identified as hot spots for interaction with TRN-SR2, the K266A and R269A single point mutants, as well as the double mutants R262A/R263A and R262A/K264A, were reported to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as blocked nuclear import.

Recently Li et al. (59) reported on the INK264E mutant that is defective for 3’ processing and strand transfer in typical in vitro assays but still displayed concerted integration activity. Unfortunately, reverse transcription by HIV-1 INK264E was reduced by 3–16-fold, obscuring further analysis. Therefore we tested the remaining alanine mutants, namely the single point mutants R262A, R263A, and K264A and the double point mutant R263A/K264A for their binding to TRN-SR2. The sin-

![FIGURE 4. Reduction in nuclear import of HIV NL4-3 INR263A/K264A. Five hours after infection with eGFP-labeled IN virus, HeLaP4 cells were fixed and analyzed by laser-scanning confocal microscopy. The ratio of nuclear/cytoplasmic green PICs was quantified (percentage nuclear PICs). A, representative slice of a stack of WT and INR263A/K264A mutant PIC virus-infected cells. The nuclear lamina was immunostained with anti-lamin A/C (red). PICs are identified as green dots, and nuclear PICs are highlighted by white arrows. The images are derived from confocal Z-stacks. B, cumulative distribution of the percentage of cells containing a certain ratio of nuclear over cytoplasmic PICs. Two independent experiments, with distinct productions of both the WT and the INR263A/K264A mutant virus, were performed (WT, n = 51 cells; INR263A/K264A, n = 49 cells; p = 0.0054, Mann-Whitney test) (WT, n = 41 cells; INR263A/K264A, n = 44 cells; p = 0.0095, Mann-Whitney test). C, nuclear import was measured in control cells (shSCR) or cells stably depleted for 75% for TRN-SR2 (shTR3). The cumulative distribution of the percentage of cells containing a certain ratio of nuclear over cytoplasmic PICs is shown for WT virus in shSCR (n = 55 cells), WT in shTR3 (n = 49 cells), INR263A/K264A in shSCR (n = 50 cells), and INR263A/K264A in shTR3 (n = 50 cells). Nuclear import of the INR263A/K264A mutant virus was significantly lower than for WT virus (p = 0.0167, Mann-Whitney test); whereas import of the WT virus in shTR3 cells was not statistically different from that of the INR263A/K264A mutant virus in shSCR cells (p = 0.5836, Mann-Whitney test). AZT, zidovudine.](image-url)
ingle point mutants R262A, R263A, and K264A did not affect TRN-SR2 binding, whereas the double point mutant R263A/K264A inhibited TRN-SR2 binding 2.3-fold (Fig. 1B). We therefore selected the INR263A/K264A mutant that interacts less with TRN-SR2 and does not perturb reverse transcription, allowing us to study the role of the TRN-SR2/IN interaction in the nuclear import of the PIC. In the past, several attempts have been made to define potential nuclear localization signals in IN. Two stretches of amino acids, 211KELQKQITK219 and 261PRRKAK266, were described as a potential bipartite nuclear localization signal (8). Our analysis of the IN/TRN-SR2 interface has allowed us to confirm the following sets of amino acids in IN: Arg262−Arg265, Lys264, and Lys266−Arg269 (27). Lysine 264 was found to be acetylated by p300 by Cereseto et al. (60). Importin 7 and importin-α3 have both been implicated as nuclear import factors for HIV through a direct interaction with IN (14, 54). Although importin 7 was later invalidated as HIV-1 nuclear import factor (11), Arg263 and Lys264 were reported as interacting amino acids. Previously, the same two IN amino acids were implicated in the interaction between IN and importin-α3 (54). In our hands, potent knockdown of importin-α3 did not affect HIV replication, bringing into question the prior claims for a significant role for importin-α3 in HIV-1 replication (Fig. 2). To assess the impact of a deficient IN/TRN-SR2 interaction on the nuclear import of HIV, the INR263A/K264A interface mutations were inserted into a molecular clone of HIV (pNL4-3), resulting in a replication-defective virus. Because in vitro analysis of the mutant integrase has evidenced a strong defect in catalytic activity, it was expected that the mutant virus does not integrate and is replication-defective. To pinpoint at which step replication is inhibited, we performed Q-PCR analyses on the different viral DNA intermediates. In parallel, we investigated the INR263A/K264A catalytic triad mutant viruses to control for the effect of defective 3′-processing on the formation of the viral DNA species. It is well known that a block in integration leads to an accumulation of two-LTR circles in the nucleus (34). Indeed, both addition of Ral during infection with WT NL4-3 and infection with NL4-3 INR263A/D116N/E152Q virus resulted on average in a 13-fold increase in two-LTR circles. Remarkably, infection with NL4-3 INR263A/K264A, which is also defective for integration, only resulted on average in a 6-fold increase in two-LTR circles. Compared with WT NL4-3, NL4-3 INR263A/K264A displayed an increased number in two-LTR circles, because of its defective integration. However, in comparison with the number of two-LTR circles for WT NL4-3 supplemented with Ral and NL4-3 INR263A/D116N/E152Q on average, a relative 2-fold decrease was measured (p = 0.01), suggesting a defect in nuclear import. This reduction may still be an underestimate of the true effect, because a lack of IN strand transfer activity (as observed for INR263A/K264A in vitro) is associated with an increase in two-LTR circle formation caused by aborted integration that may counteract the reduction in circles resulting from reduced PIC import.

Although well established, Q-PCR analysis of two-LTR circles is an indirect way to measure nuclear import of HIV. Therefore we additionally performed a cellular PIC nuclear import assay, which allows us to directly follow HIV PICs in infected cells (18, 37). In this assay, IN-eGFP is trans-incorporated into viral particles through Vpr fusion, enabling visualization by confocal microscopy. The ratio of nuclear to cytoplasmic PICs allows for direct quantification of nuclear import. Whereas two-LTR accumulation is typically measured at 24 h after infection prior to dilution by cell division, nuclear PICs are imaged by fluorescence at 5 h postinfection, because integration is believed to disassemble the PICs. Albanese et al. (37) performed a kinetics experiment to quantify the number of nuclear PICs at different time points from 3 to 24 h after infection and showed that the maximum number of intranuclear PICs was indeed reached after 6 h.

This PIC import assay further corroborated the block in nuclear import observed with the INR263A/K264A virus in comparison with WT virus. The reduction in nuclear PICs of the INR263A/K264A virus was similar to the reduction seen after TRN-SR2 depletion. When analyzing INR263A/K264A in shTR3 cells, the combination of both partial TRN-SR2 depletion and 2-fold reduced IN-TRN-SR2 interaction resulted in an additive effect on the inhibition of nuclear import. The on average 2-fold reduction in nuclear PICs and the 2-fold drop in two-LTR formation and were perfectly in line with the 2.3-fold lower binding affinity of INR263A/K264A for TRN-SR2 compared with WT IN.

We describe here how the NL4-3 virus, carrying INR263A/K264A, which is deficient for interaction with TRN-SR2, is blocked at the nuclear import step. These results directly associate HIV integrase and TRN-SR2 for a common role during HIV nuclear import. Representing a significant bottleneck for HIV replication, nuclear import is of utmost importance for successful infection of host cells. The evidence provided supports a mechanism whereby the TRN-SR2/IN interaction directly mediates nuclear import of the viral PIC, validating this protein-protein interaction as a promising target for antiviral therapy.

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