Transportin 3 and importin α are required for effective nuclear import of HIV-1 integrase in virus-infected cells

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Unlike other retroviruses, human immunodeficiency virus type-1 (HIV-1) can infect terminally differentiated cells, due to the ability of its pre-integration complex (PIC) to translocate via the host nuclear pore complex (NPC). The PIC nuclear import has been suggested to be mediated by the viral integrase protein (IN), via either the importin α or transportin 3 (TNPO3/transportin-SR2) pathways.

We show that in virus-infected cells, IN interacts with both importin α and TNPO3, simultaneously or separately, suggesting a multiple use of nuclear import pathways. Disruption of either the IN-importin α or IN-TNPO3 complexes in virus-infected cells by specific cell-permeable peptides resulted in inhibition of IN and viral cDNA nuclear import. Here we show that peptides which disrupt either one of these complexes block virus infection, indicating involvement of both pathways in efficient viral replication. Formation of IN-importin α and IN-TNPO3 complexes has also been observed in IN-transfected cultured cells. Using specific peptides, we demonstrate that in transfected cells but not in virus infected cells the importin α pathway overrides that of TNPO3. The IN-importin α and IN-TNPO3 complexes were not observed in virus-infected Rev-expressing cells, indicating the Rev protein’s ability to disrupt both complexes.

Our work suggests that IN nuclear import requires the involvement of both importin α and TNPO3. The ability to inhibit nuclear import of the IN-DNA complex and consequently, virus infection by peptides that interrupt IN’s interaction with either importin α or TNPO3 indicates that for efficient infection, nuclear import of IN should be mediated by both nuclear-import receptors.

Introduction

Integration of human immunodeficiency virus type 1 (HIV-1) cDNA into the host chromosomal DNA is one of the crucial events in the virus’s life cycle, essentially controlling the entire replication and assembly process.1 This event is mediated by the viral integrase (IN) protein which is encapsidated within virions and released into the cell cytoplasm following virus cell fusion.2 Within the cytoplasm, IN attaches to the viral cDNA, forming an IN-DNA complex which is part of the viral pre-integration complex (PIC).3 For integration to occur, the IN-DNA complex has to be translocated into the infected cells’ nuclei and reach the chromosomal DNA.4 Indeed, in contrast to other retroviruses, HIV-1 can infect terminally differentiated cells.5,6 HIV-1’s capacity to infect cell cycle-arrested cells has been ascribed to the ability of its PIC7,8 to translocate across the nuclear envelope via the nuclear pore complex (NPC).5,10 However, despite extensive effort and numerous studies, our understanding of the events leading to nuclear import of the PIC is still very poor. In particular, the detailed mechanism of the PIC’s nuclear-import pathway, as well as the viral or cellular factors that mediate its translocation into the nuclei of infected host cells, are highly controversial and hotly debated (reviewed in ref. 11–13).

Several cellular nuclear-import receptors have been suggested to be involved in the process of PIC nuclear import. Among them are the importin α/β heterodimer,14-18 importin γ19,20 and transportin 3 (TPNPO3/transportin-SR2, an importin β-like receptor).21-23 Various viral karyophilic proteins, such as the Matrix (MA), Vpr and IN,3,24-30 have been suggested to actively translocate the PIC into the host-cell nucleus. The cellular protein lens epithelium-derived growth factor p75 (LEDF/p75), along with a short section of triple-stranded DNA present within the viral cDNA and known as the DNA flap, have also been implicated in promoting translocation of the PIC into nuclei of infected cells.30-34 In addition, the HIV capsid protein (CA) has been reported to play a crucial role in controlling nuclear import of the HIV genome.35,34 However, the exact mechanism governing nuclear import of the PIC remains unclear.3,5,11,14,15,25,27,28,30

Our previous works17,18 as well as that of others,14,35-39 have demonstrated that the HIV-1 IN is a karyophilic protein that
IN interacts with both importin α and TNPO3. These results suggest that these two nuclear-import receptors are involved in the translocation of IN into nuclei of infected cells, either concurrently or alternately.

We have previously shown (reviewed in ref. 18 and see also **Figure 1B and C**) that cell-permeable peptides bearing the SV40-NLS or the putative NLS of IN (NLS-IN) can disrupt the complex formed between IN and importin α and inhibit translocation of IN into the nuclei of infected cells. As shown in **Figure 1B and C**, these two peptides failed to promote dissociation of the IN-TNPO3 complex, indicating that different domains mediate the interaction of IN with these two nuclear-import receptors. The results in **Figure 1** also show that neither peptide had any effect on the interaction between Rev and IN, shown by us previously to occur in virus-infected cells40-44 and indicating specificity of function.

**Disruption of the IN-TNPO3 complex by IN-interacting peptides blocks nuclear import of IN and viral cDNA.** Three IN-interacting cell permeable peptides were previously selected by us.53-58 These peptides, termed IN-1,53-57 Rev 53–67 (Fig. 2A and B) and Rev 13–23,46,48 (not shown respectively), have been shown to promote dissociation of the IN-TNPO3 complex but not of the IN-importin α complex. Specificity of these peptides’ functions should be inferred from results showing that a peptide bearing the NLS sequence of the Rev protein [also known as the arginine-rich motif (ARM)44,45] fails to disrupt IN interactions with either importin α or TNPO3 (Fig. 2C). However, as expected, the Rev ARM peptide was able to promote dissociation of the Rev-importin β complex (Fig. 2D). Also, and as shown previously,44 only Rev 53–67 (Fig. 2B) and Rev 13–23 (not shown) peptides promoted dissociation of the Rev-IN complex formed in virus-infected cells.
expected to lead to cytoplasmic retention of IN as well. Although the Rev-IN complex can be observed in the PICs of the infected cells, a large portion of it is localized within the cytoplasm and is not necessarily associated with the PIC. This is probably due to the fact that Rev promotes dissociation of the IN from the PIC localized viral cDNA.

The effect of the IN-interacting peptides on nuclear import of IN protein in transfected cultured cells. In light of the above and previous results observed in virus-infected cells, it was of interest to study the effect of the IN-interacting peptides on the intracellular localization of IN in transfected cultured cells. In transfected cells, as opposed to virus-infected cells, Rev 53–67 (and Rev 13–23, not shown), as well as IN-1, only partially inhibited nuclear import of the expressed IN (Fig. 3). Specificity of inhibition could be inferred from the fact that nuclear import of the Rev protein was not affected by these peptides (Fig. 5B). On the other hand, the Rev ARM peptide, as expected, blocked nuclear import of Rev but not of IN (Fig. 5A and B).
full-length Rev protein blocks IN nuclear import, it was essential to study the effect of Rev itself on the interactions between IN and the nuclear-import receptors. When Rev-expressing cells were infected with HIV-1, IN failed to interact with either importin α or TNPO3 (Fig. 6). This was inferred from the co-IP experiment showing that, in contrast to the Rev-derived peptides, the Rev protein promotes disruption of both the IN-importin α and IN-TNPO3 complexes.

Discussion

The results of the present work show that in virus-infected cells IN interacts with both importin α and TNPO3. Our previous observation showed that nuclear import of HIV-1 IN is mediated by a specific NLS domain (amino acids 161–173, NLS-IN), which mediates its translocation via the importin α pathway. 15,17,18,35 This was demonstrated by import of BSA molecules

Table 1. The effect inhibitory peptides on the extent of nuclei containing IN in infected cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of nuclei containing IN from all infected cells [%]</th>
</tr>
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<tbody>
<tr>
<td>No peptide</td>
<td>94.11 ± 3.75</td>
</tr>
<tr>
<td>IN-1</td>
<td>7.14 ± 1.26</td>
</tr>
<tr>
<td>Rev 53–67</td>
<td>5.17 ± 1.07</td>
</tr>
<tr>
<td>Rev ARM</td>
<td>72.14 ± 4.67</td>
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*The average percentage was calculated from 3 repeats of 10 random fields. For each experiment the error represent the standard deviation between each of the repeats.

Figure 3. Inhibition of IN nuclear import in virus-infected cells by Rev 53–67 and IN-1. TZM-bl cells were infected (with wild-type HIV-1) (A), incubated with the indicated peptides (B–D) and immunostained as described in Methods: IN (red), Rev (green), DAPI (blue); the entire merged picture was magnified for a better view of IN localization within the infected cell. Bar = 10 µm. Arrows indicate the presence of intranuclear IN (magenta) or Rev (cyan) molecules or Rev-IN complex (white) and of cytoplasmic IN (red), Rev (green) and Rev-IN complex (orange-yellow).

The Rev protein disrupts both IN-importin α and IN-TNPO3 complexes. Following our results regarding the ability of the Rev-derived peptides to promote dissociation of the IN-TNPO3 complex and our previous observation that the
conjugated to the NLS-IN peptides into nuclei of permeabilized cultured cells.\textsuperscript{37} Moreover, we have showed that cell-permeable peptides bearing NLS-IN, as well as those bearing SV40-NLS, were able to inhibit nuclear import of IN in transfected and virus-infected cultured cells.\textsuperscript{18} As would be expected, the same peptides inhibit HIV-1 infection of TZM-bl and lymphocyte-cultured cells.\textsuperscript{18}

Recently, our observations regarding the interaction between the viral IN and importin \(\alpha(3)\) in virus infected cells have been confirmed in independent studies using in-vitro pull-down assay and cell-based co-immunoprecipitation method in 293T cells and HIV-1 infected cells.\textsuperscript{51} Furthermore, a short hairpin RNA (ShRNA) was employed to knockdown Imp\(\alpha3\) in dividing C8166T, HeLa cells and non-dividing monocyte-derived macrophages (MDM). HIV-1 replication in Imp\(\alpha3\) knockdown cells was significantly downregulated, (reviewed in ref. 51 and 52).

The use of cell-permeable peptides allowed us to demonstrate that TNPO3, in addition to importin \(\alpha\), is required for mediation of IN nuclear import and virus replication in infected cells. However, our co-IP experiments did not allow us to determine whether IN interacts directly with TNPO3, as was demonstrated recently using an in-vitro assay system,\textsuperscript{33} or if its interaction is mediated by a third component, such as t-RNA.\textsuperscript{23} The identification of TNPO3 as a binding partner of IN has recently been confirmed independently and the IN binding site for TNPO3 has been elucidated.\textsuperscript{54}

The requirement of TNPO3 for HIV replication was based previously\textsuperscript{21-23} mainly on the significantly low level of HIV-1 replication in RNAi TNPO3-knockdown cultured cells. Our present study, demonstrating inhibition of HIV-1 infection following disruption of the IN-TNPO3 complex by the Rev-derived peptides as well as by IN-1, strongly supports involvement of TNPO3 in the HIV-1 infection process. However, the way in which TNPO3 participates in the HIV-1 replication cycle or is involved in nuclear import of the PIC is still under debate. Christ et al.\textsuperscript{21} and Luban\textsuperscript{22} suggest that TNPO3 is required to mediate nuclear import of HIV-1 IN and thus for translocation of the PIC into nuclei of infected cells and consequently, for virus infection. Our demonstration of Rev-derived and IN-1 peptides disruption of the IN-TNPO3 interaction and virus infection supports this view. On the other hand, the use of MuLV/HIV chimera viruses failed to demonstrate the requirement of IN-TNPO3 interaction for HIV replication.\textsuperscript{55}

Krishnan et al.\textsuperscript{53} used in vitro binding assays to study the interaction between TNPO3 and several IN proteins from various retroviruses, such as HIV, MuLV and SIV. A comparison of their results to the infectivity profiles of HIV chimera viruses led them to conclude that the dominant protein dictating TNPO3 dependency during virus infection is the HIV-1 CA and not IN.\textsuperscript{53} This conclusion was further supported by a work published during the preparation of this ms.\textsuperscript{45} However, no direct or indirect interaction between TNPO3 and CA has yet been demonstrated. Furthermore, rapid removal of CA from the RTC (reverse transcriptase complex)/PIC has been suggested to be required for nuclear import of the HIV-1 PIC.\textsuperscript{31} Thus, a functional interaction between TNPO3 and CA remains to be proven.

Our conclusion that nuclear import of IN requires the involvement of both importin \(\alpha\) and TNPO3 and plays a dominant role in HIV-1 infection is based mainly on results obtained using two groups of peptides (Fig. 7A). One group, which includes SV40-NLS as well as NLS-IN,\textsuperscript{18} disrupted only—and specifically—the interaction between IN and importin \(\alpha\) (Fig. 7A). The second group, which includes the two Rev-derived peptides and IN-1, disrupted only the interaction between IN and TNPO3 (Fig. 7A). Interestingly, the Rev protein which, similar to the Rev-derived peptides, has been shown to block nuclear import of IN,\textsuperscript{42} prevented interaction of IN with both receptors. The disruption of the IN-TNPO3 complex by the Rev derived and IN-1 peptides is not necessarily due to direct masking of the TNPO3 binding sites. This can also be due to the peptides inducing a change in the IN oligomeric state\textsuperscript{45-47} which may prevent binding of TNPO3.

![Figure 4. Inhibition of cDNA nuclear import and 2LTR circle formation in virus-infected cells by Rev 53–67 and IN-1. SupT1 lymphocytes were infected with wild-type HIV-1 at a MOI of 1 in the presence or absence of the indicated peptides as described in Methods. (A) Following infection, the nuclear fraction was isolated from half of the cells and the amount of viral DNA was estimated by real-time PCR. (B) The amount of 2LTR circles was estimated in the DNA fraction isolated from whole cells by real-time PCR. Other experimental details are described in Methods. Error bars represent standard deviation, ca. ±5%](image-url)
The ability to block virus infection by a peptide belonging to either the first or second group clearly indicates that for efficient infection, nuclear import of IN should be mediated at the same time by both nuclear receptors. However, it appears that in IN-expressing cells, as opposed to virus-infected cells, nuclear import via the importin-α pathway is dominant. This is inferred from the results showing significant inhibition of IN nuclear import by a peptide belonging to the first group, whereas inhibition was only partial by a peptide belonging to the second group. Thus it appears, as also indicated in our previous observations, that results obtained in transfected cells are not necessarily relevant to those obtained in infected ones.

Our results demonstrate that IN, a karyophilic protein, is translocated into the cell nucleus by two distinct and different nuclear-import pathways. Confirming previous observations, we show that blocking IN nuclear import simultaneously inhibits import of viral cDNA and the formation of 2LTR circles. These results strengthen the view that IN is an integral part of the PIC. It is thus conceivable that participation of the two nuclear-import receptors is required to allow efficient nuclear import of the relatively large PIC, whose main component is the IN-DNA complex: the function of only one of these receptors does not appear to be sufficient to drive the translocation of this IN-DNA complex via the NPC.

It is still unclear why nuclear import of IN requires the involvement of both importin-α and TNPO3. Furthermore,
we still do not know whether the same IN molecule interacts, via different domains, with both receptors or if different IN molecules—or IN oligomers—interact individually with each receptor. The scheme in Figure 7B summarizes our view of the three alternative interaction possibilities between IN and the two nuclear-import receptors importin α and TNPO3.

In addition to importin α and TNPO3, there are two additional pathways that have been implicated in PIC nuclear transport. These include the nuclear import receptor importin 7,19,20,55 and the direct interaction with Nup153,34,56 which is one of the NPC proteins.57 The current results as well as previous results20,21,34,52,58 may imply that these two pathways could also be required to ensure PIC nuclear transport as a multi nuclear import complex. Thus, inhibition of only one of these nuclear import pathways should result in partial decrease in the level of the HIV PIC nuclear import but should not block it completely.

Materials and Methods

Mammalian cells. Monolayer adherent HeLa TZM-bl cells (obtained through the NIH AIDS Research and Reference Reagent Program) expressing the β-galactosidase gene under regulation of a transactivation response element59 were grown in Dulbecco’s modified Eagle’s medium. The T-lymphocyte cell line SupT1 was grown in RPMI 1640. All media were supplemented with 10% (v/v) fetal calf serum, 0.3 g/l L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin (Biological Industries, Beit Haemek, Israel). Cells were incubated at 37°C in a 5% CO2 atmosphere and re-cultured every 4 days. IN- and Rev-expressing HeLa TZM-bl cells50 were generated by stable transfection60 into TZM-bl cells of plasmid pcDNA3.1 bearing the full-length wild-type IN or Rev gene, respectively. Selection was carried out for 4 weeks with 400 µg/ml hygromycin B.

Viruses. Wild-type HIV-1 was generated by transfection of HEK293T cells with pSVC21 plasmid containing the full-length HIV-1 HXB2 viral DNA. Wild-type viruses were harvested from HEK293T cells 48 and 72 h post-transfection. The viruses were stored at -75°C.

Virus stock titration. Quantitative titration of HIV-1 was carried out using the MAGI assay, as described by Kimpton and Emerman.61 Briefly, TZM-bl cells were grown in 96-well plates at 1 x 104 cells per well. The cells were then infected with 50 µl of serially diluted virus as described.61 Two days post-infection (PI), cultured cells were fixed and β-galactosidase was estimated exactly as described previously.61 Blue cells were counted under a light microscope at 200X magnification.

Synthesis of peptides. Peptides were synthesized on Rink amide resin using a model 433A Applied Biosystems peptide synthesizer as described.62 The Pen peptide,63 with the following sequence: RQIKIWFQNRRMKWKK (Ant 43–58), was added to the SV40-NLS- and NLS-IN-bearing peptides to allow their cell permeability as described previously.18

Immunostaining studies. Localization of IN and Rev in transfected cultured cells. TZM-bl IN- or Rev-expressing cells were grown on chamber slides (Nunc). After reaching 70–80% confluence, cell cycle arrest was obtained by treatment with 5 µg/ml of aphidicolin. The cells were then incubated with 150 µM of the indicated peptide for 6 h and fixed and immunostained.
essentially as described previously with the following modifications. After fixation, cells were blocked with 5% (w/v) BSA (IgG free) (Jackson) in PBS for 60 min. For detection of HIV-1 IN, cells were incubated with 1:100 rabbit α-IN (NIH AIDS Research and Reference Reagent Program, cat. no. 758) or with 1:50 α-Rev at room temperature for 60 min. Then the cells were incubated with a second antibody, Cy3-conjugated anti-rabbit antibody (Jackson) (1:200) or Cy2-conjugated anti-rat antibody (Jackson) (1:200) at room temperature for 60 min, followed by another five washes with PBS + 0.05% Tween 20. For detection of DNA, cells were stained with DAPI according to the manufacturer’s protocol (KPL, USA). The slides were prepared with mounting media (Bio-Rad) and immunofluorescent cells were detected with a confocal microscope.

Localization of IN and Rev in HIV-1-infected cultured cells. TZM-bl cells were grown on chamber slides. Cells were arrested as described in A and then incubated with 150 µM of the indicated peptide for 2 h. After incubation with the peptides, cells were infected with wild-type HIV-1 at a multiplicity of infection (MOI) of 25. Cells were fixed and stained as described above with the following modifications: fixation was performed at 12 h PI and the first antibody was used at a dilution of 1:50, the second antibody at a dilution of 1:100 for the detection of IN. For the detection of Rev, the first antibody was used at a dilution of 1:50, the second antibody at a dilution of 1:50.

In both cases 10 fields were selected from each experiment and one shown is a representative of the image obtained.

Co-IP studies of in-vivo protein-protein interactions. Cells were infected with the indicated viruses at a MOI of 15, harvested at 12 h PI, washed three times with PBS and lysed by the addition of PBS containing 1% (v/v) Triton X-100. Half of the lysate volume was subjected to SDS-PAGE, then immunoblotted with either antiserum raised against IN amino acids 276–288 (anti-IN) (NIH AIDS Research and Reference Reagent Program, cat. no. 758), anti-importin α (anti-Impα) antibody (Santa Cruz), anti importin β (anti-Impβ) antibody (Santa Cruz), anti-TNPO3 antibody (Abcam) or anti-Rev antibody. The complementary HRP-conjugated antibodies (Jackson) were used as the second antibody.

The remaining lysate or isolated fractions were incubated for 1 h at 4°C with anti-Impα, anti-Impβ, anti-TNPO3, anti-IN or anti-Rev antibodies. Following 3 h incubation with protein G-agarose beads (Santa Cruz) at 4°C, the samples were washed three times with PBS containing 1% (v/v) Nonidet P-40. SDS buffer was added to the samples and after boiling and running on an SDS polyacrylamide gel, the membranes were immunoblotted with anti-Impα, anti-Impβ, anti-TNPO3, anti-IN or anti-Rev antibodies and then with the complementary HRP-conjugated antibodies (Jackson) as second antibodies.

When peptides were used, cells were incubated with 150 µM of the indicated peptide for 2 h prior to infection.

Isolation of cytoplasm and nuclei from infected cells. The various fractions were obtained from virus-infected cells essentially as described previously with several modifications. Briefly, cells were harvested and washed twice in buffer A (20 mM Hepes pH 7.3, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT and 0.1 mM PMSF). Cells were then suspended in 200 µl of buffer A containing 0.025% (w/v) digitonin, incubated at room temperature for 10 min and then centrifuged for 3 min at 1,000 g at room temperature. The supernatant was then centrifuged at 8,000 g and separated into supernatant (cytoplasm) and pellet (nuclei) and stored at -70°C.

Quantitation of total and nuclear viral DNA. Total viral DNA was estimated, using SYBR green real-time quantitative PCR at 10 h PI, from the total cell lysate or nuclear-isolated fractions of the infected cells. DNA was isolated by phenol chloroform method. DNA samples (1 µg) were added to 95 µl containing 1X Hot-Rescue Real Time PCR Kit-SG (DiaThera s.r.l. Fano, Italy) and 100 nM of each primer-binding site primer: F5 (5’ primer, 5'-TAG CAG TGG CGC CCG A-3’) and R5 (3’ primer, 5’-TCT CTC TGC TCC TAG CCT CCG C-3’). All amplification reactions were carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) under the following program: 1 cycle at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 35 s at 68°C. Three replicates were performed for each PCR run. All other details are exactly as described in Casabianca et al.

Quantitation of 2LTR circles. Quantification of 2LTR circles was estimated exactly as described in Butler et al.

All experiments were repeated three to four times and the quantitative differences between the experiments never exceeded ±10%.

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Authors’ Contributions
A. Levin designed and performed the experiments, analyzed data and contributed to writing the paper; Z.H. performed peptide synthesis and purification; A.F. designed the study and contributed to the writing; A. Loyter designed the study, contributed to the writing of the paper and coordinated the study. All authors have read and approved the manuscript.
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