HIV-1 Infection Requires a Functional Integrase NLS

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Summary

HIV-1 is able to infect nondividing cells productively in part because the postentry viral nucleoprotein complexes are actively imported into the nucleus. In this manuscript, we identify a novel nuclear localization signal (NLS) in the viral integrase (IN) protein that is essential for virus replication in both dividing and nondividing cells. The IN NLS stimulates the efficient nuclear accumulation of viral DNA as well as virion-derived IN protein during the initial stages of infection but is dispensable for catalytic function. Because this NLS is required for infection irrespective of target cell proliferation, we suggest that interactions between uncoated viral nucleoprotein complexes and the host cell nuclear import machinery are critical for HIV-1 infection of all cells.

Introduction

One of the hallmarks that distinguishes human immunodeficiency virus type-1 (HIV-1) and other lentiviruses from oncoretroviruses is their capacity to infect nondividing cells productively (Weinberg et al., 1991; Lewis et al., 1992). This feature may be particularly important in exposed hosts, where infection of postmitotic cells such as tissue macrophages and mucosal dendritic cells as well as of nondividing T cells may be essential not only for viral transmission and dissemination, but also for the establishment of persistent viral reservoirs. At the molecular level, this dichotomy between HIV-1 and oncoretroviruses has been attributed to the karyophilic properties of HIV-1 postentry nucleoprotein complexes (these are often called preintegration complexes, or PICs). According to this model, it is assumed that HIV-1 PICs harbor one or more nuclear localization signals (NLSs) that mediate import across the intact nuclear envelope via the nuclear pore complexes (NPCs) (Bukrinsky et al., 1992). In the case of oncoretroviruses such as murine leukemia virus (MLV), it has been shown that the integration of viral DNA into the host cell genome requires mitosis (Roe et al., 1993; Lewis and Emerman, 1994). This suggests that MLV PICs may lack NLSs and that it is the transient disassembly of the nuclear envelope that occurs during mitosis which enables these complexes to accumulate in the nucleus.

Based on this view of MLV infection, it has been suspected that productive infection of dividing cells by HIV-1 may still take place in the absence of NLS-mediated PIC import. In other words, the prediction is that HIV-1 PIC NLSs may be selectively required for nondividing cell infections. Alternatively, it is also possible that HIV-1 may not follow the MLV paradigm and that infection of all cell types may depend on the translocation of PICs through NPCs. To address these questions and to learn more about the early steps in HIV-1 infection, it is necessary to delineate the karyophilic signals present within PICs.

Although a detailed compositional and stoichiometric description of HIV-1 PICs, and in particular of the complexes that actually traverse the nuclear envelope, has yet to be attained, a number of viral and cellular proteins have been identified as being associated with these multicomponent complexes (reviewed by Fouchier and Malim, 1999). Among the viral proteins, the integrase (IN), matrix (MA, p17 Gag), nucleocapsid (NC, p7 Gag), Vpr, and reverse transcriptase (RT) gene products have been found in association with partially purified PICs. Although MA, Vpr, and IN have each been reported as having karyophilic properties (Bukrinsky et al., 1993; Heinzinger et al., 1994; Gallay et al., 1995, 1997; Fouchier et al., 1998; Vodicka et al., 1998; Pluymers et al., 1999), the accurate definition of their NLSs and the elucidation of the role(s) of such sequences in virus infection continues to be controversial. Indeed, in our view, a bona fide protein-based NLS that plays an essential (as opposed to moderating) role in PIC nuclear import has not yet been defined.

In this report, we define a noncanonical NLS within the catalytic core domain of HIV-1 IN that is essential for productive infection of all cell types. We also show that this NLS is required for the efficient postentry nuclear localization of both virion-associated IN protein and newly synthesized reverse transcripts. We propose that the NLS of IN is required for the appropriate nuclear import of HIV-1 PICs and suggest that the translocation of these nucleoprotein complexes through NPCs is a required step for the infection of both dividing and nondividing cells.

Results

The size limit for passive diffusion through NPCs is ~50 kDa for a globular protein, or ~9 nm (reviewed by Mattaj and Englmeier, 1998). Given the multicomponent nature of HIV-1 PICs and their estimated Stokes radius of ~28 nm (Miller et al., 1997), it is evident that their import into
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Figure 1. HIV-1 Integrase Encodes a Transferable NLS

(A) Subcellular localization of PK-IN fusion proteins. HeLa cells expressing chimeric proteins comprising Myc-tagged PK at the amino terminus and the indicated regions of IN at the carboxyl terminus (b)–(g) were analyzed by immunofluorescence.

(B) The three-domain structure of HIV-1 IN. The sequence of the 161–173 region of the HIV-1YU-2 isolate is shown (this is the consensus for clade B isolates [Kuiken et al., 1999]), and the two residues essential for NLS function are indicated (in black).

the nucleus must be signal mediated. To identify peptide sequences important for this process, we have been delineating NLSs in individual PIC proteins, introducing inactivating mutations into proviral clones, and then studying the ensuing infectivity phenotypes.

HIV-1 Integrase Encodes an NLS

To identify protein components of the HIV-1 PIC with nuclear import activity, fusions of MA, NC, RT, IN, or Vpr to the carboxyl terminus of pyruvate kinase (PK) were evaluated for subcellular localization in transfected HeLa cells. As previously established, PK, a protein of \( \sim 55 \) kDa, exceeds the diffusion limit of NPCs and is cytoplasmic (Figure 1Aa) (Fouchier et al., 1997). The fusion with IN localized to the nucleus (Figure 1Ab), the fusion with Vpr accumulated throughout the nucleus and the cytoplasm (data not shown), and the MA, NC, and RT chimeras were each detected in the cytoplasm (data not shown) (Fouchier et al., 1997). We concluded that HIV-1 IN harbors a functional NLS and therefore pursued studies aimed at defining its role in PIC nuclear import. Notably, the demonstration that HIV-1 IN encodes an NLS is consistent with earlier results describing the import of a glutathione S-transferase (GST)-IN fusion in microinjected cells (Gallay et al., 1997) or a green fluorescent protein (GFP)-IN fusion in transfected cells (Pluymers et al., 1999).

In the initial mapping of the IN NLS, many in-frame fusions of IN fragments to PK were examined; the most informative results are shown in Figure 1A. Analysis of carboxy-terminal truncations indicated that nuclear targeting capability lies between residues 152 and 184 (Figures 1Ac and 1Ad), whereas results obtained with amino-terminal truncations showed that nuclear localization mapped between residues 161 and 174 (Figures 1Ae and 1Af). Consistent with these findings, a 13-amino acid region spanning residues 161–173 conferred efficient nuclear localization to PK, thus defining this region as a bona fide NLS (Figure 1Ag). Figure 1B shows the amino acid sequence of the NLS and its placement within the domain structure of IN (reviewed by Esposito and Craigie, 1999). It is located toward the carboxyl terminus of the catalytic core domain but outside of the catalytic triad of aspartic acid and glutamic acid residues [the D,D(35)E motif]. Moreover, examination of the three-dimensional structure of the catalytic core (Dyda et al., 1994; Chen et al., 2000) reveals that the NLS is located around a surface-exposed loop that joins \( \alpha \) helices 4 and 5, a feature that could facilitate interactions with cellular factors.

Definition of Critical Residues within the IN NLS

To address the role of IN nuclear localization in HIV-1 infection, it was important to identify NLS-inactivating mutations within IN that were minimally disruptive to overall structure. Comparison of the IN NLS (Figure 1B) with known NLSs revealed little in the way of sequence similarity (Mattaj and Englmeier, 1998); for instance, unlike many NLSs, this sequence lacks a preponderance of basic residues. All residues between positions 161 and 173 (except residue 169) were therefore subjected to alanine-scanning mutagenesis, and NLS function was determined in HeLa cells using the PK-IN(161-173) vector (Figure 2A). Of these 12 alanine substitution mutants,
The defined IN NLS of HIV-1 YU-2 (a clade B virus) is aligned with the corresponding regions of IN proteins of other HIV-1 clades, HIV-2, and SIVs drawn from five phylogenetic lineages of primate immunodeficiency viruses. The sequences for clades A, B, C, and D of HIV-1, HIV-2 (clade A), SIV/SMM (sooty mangabey), as well as SIV/AGM(VER) and SIV/AGM(SAB) (African green monkey, vervet, and sabaesu) represent consensuses, whereas those for HIV-1YU-2, SIV/CPZ(US), SIV/SYK, and SIV/L’HOEST are from individual isolates (Kuiken et al., 1999). The area boxed in gray identifies the residues that align with V165 and R166.

Interestingly, the two residues that we find to be essential for NLS activity appear to be well conserved among these viruses; the arginine at position 166 is absolutely conserved, and the only variation at position 165 is the conservative exchange for isoleucine. Only the loss of the valine at position 165 (the V165A mutation) or the arginine at position 166 (the R166A mutation) abolished nuclear import (compare Figures 2Ab and 2Ac with Figure 2Ad). In contrast, disruption of adjacent residues, such as the glutamine at position 164, had no discernible effect (Figure 2Aa).

In addition to IN fusion proteins, the ~32 kDa HIV-1 IN protein also accumulates in the nucleus when expressed as a nonfusion (Jones et al., 1991; Petit et al., 1999). Using an epitope-tagged version of IN, we were able to confirm this observation in transfected HeLa cells (Figure 2Bd). As above, we found that the integrity of residues 165 and 166, but not of residue 164, was essential for efficient nuclear targeting in this context; Western blot analysis confirmed that these mutant and wild-type IN proteins were expressed at similar levels (Figure 2C). The finding that small fractions of the V165A and R166A proteins accumulated in the nucleus may be attributable either to the presence of additional (relatively weak) NLSs elsewhere within IN or to passive diffusion. These experiments, therefore, identify a discrete NLS between residues 161 and 173 of HIV-1 IN that is both necessary and sufficient for nuclear localization. This sequence is not the first region of IN to have been assigned a role in nuclear import. For example, the lysine at position 186, the glutamines at positions 214 and 216, and the cysteine at 130 have, when disrupted, each been shown to compromise the nuclear accumulation of IN (Gallay et al., 1997; Petit et al., 1999, 2000). In these cases, however, the presence of a transferable NLS was not demonstrated, and it has been suggested that disruptions to protein conformation, rather than NLS inactivation, may have caused the loss of nuclear import (Petit et al., 2000).

A sequence alignment of the 161–173 region of HIV-1 YU-2 IN with the corresponding regions of other HIV and SIV IN proteins reveals very strong sequence conservation among the HIV-1/SIVy2 viruses, but rather less when compared to HIV-2 and the other SIVs (Figure 3). Interestingly, the two residues that we find to be essential for NLS activity appear to be well conserved for these viruses; the arginine at position 166 is absolutely conserved, and the only variation at position 165 is the conservative exchange for isoleucine.

Inactivation of the IN NLS Does Not Impart Pleiotropic Loss of Function
To evaluate the role of the IN NLS during HIV-1 infection, the V165A and R166A inactivating mutations as well as the proximal noninactivating Q164A and D167A mutations were each introduced into the macrophage-tropic isolate HIV-1 YU-2. Prior to analyzing infection phenotypes, it was important to determine whether NLS disruption resulted in broad perturbations to IN function, perhaps through protein misfolding. For instance, in addition to mediating integration itself, the integrity of IN is also important for virus assembly and morphogenesis, as well as for efficient reverse transcription (reviewed by Engelman, 1999).

Mutant virus stocks produced by transfection of 293T cultures contained similar levels of p24Gag and RT activity as did the wild-type virus, suggesting that there were no gross assembly or budding defects (data not shown). Western blot analysis of virus-producing cell lysates and supernatant virions further showed that the V165A and R166A mutations did not appear to affect the proteolytic
As a further marker for the structural integrity of IN core domains, we measured catalysis in vitro using purified recombinant proteins and a "disintegration" assay where activity is manifested as the formation of a covalent bond between two synthetic oligonucleotides (Figure 4C). Both NLS-deficient IN proteins were as active as the wild-type protein, as evidenced by the slower-migrating species in lanes 3–5. As expected, a protein carrying a missense mutation in the catalytic triad (D64A) was completely inactive (Figure 4C, lane 2). Together, these assays indicate that many of the known functions and attributes of IN are unaffected by NLS inactivation.

The IN NLS Is Required for Infection of Dividing and Nondividing Cells

The importance of the IN NLS for HIV-1 replication was tested by challenging a variety of dividing (peripheral blood mononuclear cells and the T cell lines CEM-SS/CCR5 and C8166/CCR5) and nondividing (monocyte-derived macrophages and microglia) cells with wild-type or mutant viruses and measuring the time-dependent accumulation of p24Gag in culture supernatants (representative results are summarized in Table 1). The outcome of multiple experiments was very straightforward, in that we never detected replication for viruses harboring a nonfunctional IN NLS (V165A or R166A viruses) regardless of target cell proliferation; specifically, supernatant p24Gag levels were always less than 10 pg/ml. In contrast, viruses carrying non-NLS-disrupting mutations in adjacent residues (Q164A or D167A viruses) replicated efficiently in all cells tested (data not shown).

To measure infectivity in a more quantitative fashion, we then turned to single-cycle challenges of reporter cell lines (Table 1). In each line, a reporter gene is under the transcriptional control of an HIV LTR such that expression is induced by HIV-1 infection as a consequence of viral DNA synthesis and ensuing viral Tat production. First, proliferating MAGI/CCR5 cells (where the β-galactosidase gene serves as the reporter) were challenged (lanes 1–4, and lanes 5–8 lower right panel) or IN-specific (lanes 5–8 with each virus, and infectivity was judged as the number of cells in which β-gal was visibly induced. The V165A or R166A viruses were reduced in infectivity by more than 100-fold (the presence of Vpr also proved to be inconsequential). Whether the low levels of β-gal that were induced by the IN NLS-deficient viruses reflected low levels of integration or a modicum of integration-independent Tat expression (Stevenson et al., 1990; Wiskerchen and Muesing, 1995) is addressed below (Figure 5A).

We next used CCR5-expressing GHOST cells (where the reporter is the gene for GFP) to determine whether cell division influences the infection-mediated induction of reporter gene activity. Proliferating MAGI/CCR5 cells (where the β-galactosidase gene serves as the reporter) were challenged with each virus, and infectivity was judged as the number of cells in which β-gal was visibly induced. The V165A or R166A viruses were reduced in infectivity by more than 100-fold (the presence of Vpr also proved to be inconsequential). Whether the low levels of β-gal that were induced by the IN NLS-deficient viruses reflected low levels of integration or a modicum of integration-independent Tat expression (Stevenson et al., 1990; Wiskerchen and Muesing, 1995) is addressed below (Figure 5A).

We next used CCR5-expressing GHOST cells (where the reporter is the gene for GFP) to determine whether cell division influences the infection-mediated induction of reporter gene activity. Proliferating or γ-irradiated G2-arrested GHOST cultures were challenged, and the percentages of cells expressing GFP were determined by flow cytometry (note that the IN NLS-deficient viruses were used at 100-fold higher inocula). Comparison of the results reveals that the relative infectivities of wild-type, NLS-deficient, or Δvpr viruses were unaffected by the loss of target cell proliferation. In contrast, and as expected, the infectivity of the oncoretrovirus MLV was ~30-fold lower in challenges of nondividing cells. These results show that the IN NLS is essential for HIV-1 infection and replication regardless of cell division; in other
Table 1. Role of the IN NLS in Virus Replication and Single-Cycle Infectivity

<table>
<thead>
<tr>
<th>Virus</th>
<th>Replication</th>
<th>Single-Cycle Infectivity</th>
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<tbody>
<tr>
<td></td>
<td>CEM-SS/CCR5</td>
<td>Primary Microglia</td>
<td>Blue Cells (MAGI)</td>
<td>% GFP Expression (GHOST)</td>
</tr>
<tr>
<td></td>
<td>(Dividing)</td>
<td>(Nondividing)</td>
<td>per ng p24Gag</td>
<td>(Dividing) (Nondividing)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>+</td>
<td>3200</td>
<td>26.2</td>
</tr>
<tr>
<td>Δvpr</td>
<td>+</td>
<td>+</td>
<td>2900</td>
<td>25.2</td>
</tr>
<tr>
<td>V165A</td>
<td>–</td>
<td>–</td>
<td>7.8</td>
<td>8.7</td>
</tr>
<tr>
<td>V165A/Δvpr</td>
<td>ND</td>
<td>ND</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>R166A</td>
<td>–</td>
<td>–</td>
<td>5.7</td>
<td>11.4</td>
</tr>
<tr>
<td>R166A/Δvpr</td>
<td>ND</td>
<td>ND</td>
<td>5.6</td>
<td>8.8</td>
</tr>
<tr>
<td>MLV/tat</td>
<td>ND</td>
<td>ND</td>
<td>20.1</td>
<td>0.68</td>
</tr>
<tr>
<td>Wild-type + AZT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.3</td>
</tr>
<tr>
<td>Mock</td>
<td>ND</td>
<td>ND</td>
<td>0.19</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Virus replication assays were maintained for 2 to 3 weeks, and virus production was monitored as supernatant p24Gag levels. In contrast to the robust replication of viruses with wild-type IN, neither IN NLS-deficient virus ever yielded detectable replication in dividing or nondividing cells. Similar results were also obtained using cultures of C8166/CCR5 cells, peripheral blood mononuclear cells, and monocyte-derived macrophages. ND, not determined.

* Numbers of β-gal-expressing MAGI/CCR5 cells were counted 24 hr postinfection and adjusted to the input inocula.

* Percentages of GFP-expressing GHOST-X4/R5 cells were determined by flow cytometry 48 hr after challenge. The values that are underlined (V165A and R166A viruses) were obtained with 100-fold higher inocula.

words, the karyophilic properties of IN are not selectively required for infection of nondenuding cells.

**Impaired Nuclear Accumulation of Viral DNA in the Absence of the IN NLS**

We next wished to determine the nature of the infectivity block(s) for the V165A and R166A viruses. In the first series of experiments, we examined viral DNA synthesis and fate during the first 24 hr of infection (Figure 5): T cells were therefore challenged at relatively high inocula, and low and high molecular weight DNAs were extracted after 7 or 24 hr.

First, we used *Alu*-PCR to measure the abilities of wild-type virus, the IN NLS-deficient viruses, and a virus carrying the D64A mutation to mediate provirus formation. Because this assay employs primers that match the viral LTR or the repetitive *Alu*-elements that are present throughout the human genome, amplicon generation is dependent on the covalent joining of viral DNA to the target cell genome. Figure 5A shows the analysis of PCR products obtained using high molecular weight DNAs prepared at 24 hr; a degree of quantitation was achieved through serial dilution of the DNA samples prior to amplification.

As expected for the wild-type virus, efficient integration at multiple sites was visualized as a broad “smear” at all dilutions tested (Figure 5A, lanes 11–13). In contrast, only barely detectable signals were noted for the undiluted samples derived from the D64A, V165A, and R166A challenges (Figure 5A, lanes 3–10). These data indicate that integration in living cells is at least 1000-fold more efficient for the wild-type virus than for these mutants. In fact, we suggest that the weak signals detected in lanes 4, 7, and 10 may actually represent low levels of nonhomologous recombination rather than inefficient IN-mediated integration (Gaur and Leavitt, 1998). In light of these results and indications that the V165A and R166A proteins retain catalytic activity (Figure 4C), we suspected that the NLS-deficient viruses may be impaired for a step in infection that precedes (and is distinct from) provirus formation.

To characterize this preintegration defect(s) further, the low molecular weight DNAs were analyzed for the presence of full-length reverse transcripts and 1- and 2-LTR circles by restriction digestion and Southern hybridization (Figure 5B). Because the cellular activities that mediate retroviral DNA circularization are considered to be nuclear, these dead-end by-products of cDNA synthesis are used as a marker of PIC nuclear import (Brown et al., 1987). The first conclusion that can be drawn from these data is that initial cDNA synthesis was not significantly impaired by loss of the IN NLS (Figure 5B, compare lanes 1 and 3 with lane 7). The second major point is that the accumulation of circular viral DNAs at 24 hr was substantially diminished for the V165A and R166A viruses (Figure 5B, compare lanes 2 and 4 with lanes 6 and 8). In the case of comparisons with the wild-type sample, the differences in LTR-circle levels are likely to result in the overestimation of relative nuclear accumulation, since a proportion of wild-type full-length cDNAs but not NLS-deficient DNAs become integrated and cannot serve as substrates for circularization.

These analyses show that the IN NLS is essential for provirus formation in vivo and that its integrity stimulates the nuclear accumulation of viral cDNAs. It is clear that LTR-circle formation and, by extension, PIC nuclear import was not completely ablated for the V165A and R166A viruses. Given that the levels of viral DNA present by 24 hr were significantly lower for these viruses (Figure 5B), we assume that loss of the IN NLS results in the accelerated turnover of unintegrated cDNA. Although this degradation could take place in the nucleus, we consider it probable that it occurs in the cytoplasm because of impaired PIC nuclear import (see below).

In view of the extremely low levels of integrated viral DNA seen for the V165A and R166A viruses (Figure 5A), we also conclude that the minor inductions of reporter gene activity that were observed for these viruses (Table 1) reflect inefficient Tat expression from LTR circles. Consistent with this assertion, the D64A virus, with its higher levels of unintegrated nuclear DNA, yielded ~4-fold higher numbers of GFP-expressing cells in GHOST cell challenges than did NLS-deficient viruses (data not shown).
Figure 5. Reverse Transcription and Viral cDNA Fate in Infected T Cells

G2-arrested CEM-SS/CCR5 cells were challenged with wild-type or IN mutant virus, and low and high molecular weight DNAs were isolated at 7 or 24 hr.

(A) Provirus formation. High molecular weight DNAs extracted 24 hr postinfection were diluted in 10-fold increments, subjected to Alu-PCR, and analyzed by Southern hybridization. The negative controls were: lane 1, no DNA template; lane 2, DNA from mock-infected cells.

(B) DNA synthesis and nuclear accumulation. Low molecular weight DNAs were digested with BsgI and subjected to Southern analysis (3-fold more DNA was present in the 7 hr samples than in the 24 hr samples). The bands corresponding to the linear, 1-LTR circle, or 2-LTR circle forms of viral DNA are indicated; the diagram explains their derivation. Identical results were obtained for both assays [A] and [B]) using proliferating cell targets.

HIV-1 IN Mediates Nuclear Import during Infection

To address the defect for the IN NLS-deficient viruses further, the nuclear import of virion-associated proteins was examined by subcellular fractionation of newly infected cells (Figure 6). T cells were challenged with the V165A, R166A, or wild-type viruses, incubated for ~90 min, and then separated into four distinct samples. These represented: insoluble material comprising nuclear and cytoplasmic components such as the cytoskeleton (In), a soluble nucleoplasmic fraction (Ns), an insoluble cytoplasmic fraction (Ci), and soluble cytosol (Cs) (refer to Figure 6A, lanes 13–16 for controls). The localization of wild-type or mutant IN proteins to these fractions was determined by Western blot analysis of either immunoprecipitates or solubilized pellets. Importantly, we consider IN to be the preferred protein to monitor in experiments of this genre because of its obligate presence in, and tight association with, PICs (Farnet and Haseltine, 1991; Miller et al., 1997).

In the wild-type infection, IN was efficiently translocated to the soluble nuclear fraction (Figure 6A, lanes 1–4), a finding that is consistent with previous analyses (Gallay et al., 1995; Bukrinskaya et al., 1996). In contrast, the IN proteins of both NLS-deficient viruses accumulated predominantly in the insoluble fraction, with only relatively minor amounts being detected in the nuclear fraction (Figure 6A, lanes 5–12). For biochemical analyses such as these, it is useful to validate that the viral proteins being measured are entering the cell by physiological means. In particular, up to 90% of the HIV-1 antigen present in challenged cells can enter nonspecifically via endocytosis (Maréchal et al., 1998). Because we used a virus that requires CCR5 for entry, the endocytic contribution to virion uptake can be evaluated by comparing cells that do or do not express this coreceptor.
Segregation of the Nuclear Import and Integration Functions of HIV-1 IN

To this point, our data suggest that the IN NLS is required for correct nuclear targeting of the PIC. However, even though the V165A and R166A proteins display catalytic function in vitro (Figure 4C), it remained possible that these mutant proteins could be integration defective in the context of infection. In addition, the finding that the low levels of viral DNA that appear in the nucleus during an NLS-deficient infection are unable to form proviruses (Figure 5) needed to be considered. To address these issues, we exploited a system in which IN proteins are incorporated into virions in trans as fusions to Vpr, such that complementation of mutations in proviral (or cis) IN can be achieved (Figure 7A) (Fletcher et al., 1997; Wu et al., 1997). Specifically, we asked whether supplying an NLS-deficient IN mutant in trans could restore infectivity to the catalytically deficient D64A virus.

Virus stocks were generated using the D64A provirus and expression vectors for Vpr-RT-IN chimeras that included the wild-type, D64A, or V165A IN proteins. Western blot analysis of virions produced in the presence of a protease (PR) inhibitor confirmed that these fusion proteins were packaged equivalently (inclusion of the PR inhibitor enabled trans IN to be distinguished from cis IN) (Figure 7B). Viruses produced in parallel but in the absence of an inhibitor were then used in T cell challenges. Cell lysates were prepared at 20 hr and analyzed by Alu-PCR to measure integration (Figure 7C). As before, the D64A virus gave rise to minimal signals either on its own or with additional D64A protein supplied in trans (Figure 7C, lanes 1, 2, 5, and 6). However, and most importantly, provision of the V165A protein as a Vpr fusion stimulated provirus formation with an efficiency close to that of wild-type IN (Figure 7C, compare lanes 3 and 4 with lanes 7 and 8).

We conclude that the NLS-deficient V165A IN protein is catalytically active in the context of HIV-1 infection. Presumably, this activity can only be manifested when the PIC reaches its required nuclear destination; for the virus that contains a mixture of the V165A and D64A proteins, we infer that these targeting functions are provided by the D64A protein. In other words, the integration and nuclear import activities of HIV-1 IN appear to be discrete functions.

Discussion

In this manuscript, we describe the identification of a conserved 13-amino acid nonbasic type NLS within the IN protein of HIV-1. Inactivation of this NLS in the context of an infectious proviral clone (the V165A and R166A mutations) revealed that this motif is essential for provirus formation and, consequently, for virus replication (Table 1 and Figure 5A). By analyzing infected cells within 24 hr of viral challenge, we found that the NLS of virion-associated IN plays an important role in the postentry nuclear accumulation of both newly synthesized reverse transcripts and the IN protein itself (Figures 5B and 6). Finally, control studies demonstrated that disruption of the NLS does not inactivate many of the known activities and attributes of IN. Specifically, catalytic activity in vitro and in living cells, virion production and the incorporation of IN, the subcellular localization and processing of viral polyproteins, IN multimerization, and reverse transcription were largely unaffected by alanine substitutions within the central valine-arginine dipeptide (Figures 4, 5B, and 7, and data not shown). Accordingly, we have concluded that the NLS of HIV-1 IN is a critical peptide mediator of PIC nuclear import and that this function is required for productive viral infection of dividing and nondividing cell targets.
The phenotype of an IN NLS-deficient virus is reminiscent of that of a virus with an inactivated central purine tract (cPPT) (Zennou et al., 2000). In addition to being noninfectious, both classes of mutant are able to complete reverse transcription but are debilitated in their ability to accumulate LTR circles (Figure 5B). One attractive explanation for this confluence of data is that the NLS of IN and the region of plus-strand viral DNA overlap (this triple-stranded structure is called the central DNA flap) that is formed proximal to the cPPT may act as a composite NLS to recruit cellular factors necessary for efficient PIC import. Clearly, elucidating more precisely the mechanism by which these peptide and nucleic acid targeting signals may coordinate their activities will require identification of the relevant interacting factors and nuclear transport receptors. Interestingly, there are precedents for nucleoprotein complexes exploiting a combination of peptide and nucleic acid targeting signals to achieve nuclear import: for example, the import of snRNPs is dependent on the 5'-trimethyguanosine (m3G) cap and a signal formed by the Sm core proteins (Huber et al., 1998).

IN is not the only HIV-1 PIC protein to have been ascribed postentry nuclear import function. The first was MA, where a short stretch of basic amino acids was described as being selectively important for infection of nondividing cells (Bukrinsky et al., 1993; Gallay et al., 1995). In contrast, more recent studies have shown that viruses lacking the majority of MA or carrying mutations within this basic motif retain substantial levels of infectivity in both dividing and nondividing cell targets (Foucher et al., 1997; Reil et al., 1998). Moreover, others have shown that this patch of basic residues is important for at least one other aspect of replication, namely the association of p55Gag with the plasma membrane during virus assembly (reviewed by Freed, 1998). These observations, coupled with our inability to show NLS function for MA or its basic motif (Foucher et al., 1997), indicate that this region of MA may only be a minor contributor to PIC import. The other PIC protein to be identified as a facilitator of PIC import was Vpr. In this case, it is clear that Vpr has nuclear import capabilities and that Vpr can interact with host proteins related to nuclear transport such as importin-α and certain nucleoporins (Foucher et al., 1998; Popov et al., 1998; Vodicka et al., 1998). Although Table 1 does not show an effect of Vpr on virus infectivity, many studies using a wide range of cell types have demonstrated that Vpr potentiates (but is not essential for) PIC import and virus replication (Heinzinger et al., 1994; Foucher et al., 1998; Vodicka et al., 1998). A possible explanation for such phenotypic variation is that Vpr's ability to stimulate PIC import may depend on host import factors that are subject to cell type-specific regulation. In sum, we conclude that residues 161–173 of HIV-1 IN constitute the peptide NLS that is most important for PIC nuclear transport. The contributions of Vpr and MA appear, therefore, to be modulatory rather than essential.

Although the NLS of IN is essential for productive infection and provirus formation (Table 1 and Figure 5A), our experiments indicate that some PIC nuclear import can take place for IN NLS-deficient viruses. Specifically, whereas infections with the V165A and R166A viruses yielded no significant signals by Alu-PCR (Figure 5A), low levels of reporter gene induction, LTR circle accumulation, and postentry nuclear-localized IN were noted (Table 1; Figures 5B and 6A). We propose that these findings have at least two implications. First, that IN NLS-deficient PICs still enter the nucleus implies that other karyophilic signals within these nucleoprotein complexes can mediate inefficient import; the principal candidate for such a signal would be the central DNA flap. Second, the inability of IN NLS-deficient viruses to integrate in vivo indicates that this peptide sequence may have an additional activity that is operative between translocation across the nuclear envelope and integration itself. Because the V165A mutant protein retains integration function in vivo (when copackaged with the D64A protein; Figure 7C), we speculate that this function may be the intranuclear trafficking of PICs to the sites of integration in host cell chromatin, or even the attachment of these complexes to chromatin. In this regard, it is provocative that two chromatin-associated proteins, In1 and HMGI(Y), have been shown to stimulate integration in vitro and to bind to HIV-1 IN or to associate with HIV-1 PICs (Kalpana et al., 1994; Farnet and Bushman, 1997).

One of the important ideas to emerge from this work is that the signal-mediated nuclear import of HIV-1 PICs appears to be essential for productive infection of both nondividing and dividing cells. This conclusion is at variance with the prevailing dogma that nuclear import is selectively required for nondividing cell infections, and that NLS-deficient viruses can exploit nuclear envelope breakdown to achieve localization to the nuclear interior. That this latter process is not capable of complementing NLS-deficient HIV-1 infections raises a number of interesting issues and questions. First, if mitosis does not enable import-deficient HIV-1 mutants to undergo productive infection, then why does it allow infection by MLV? We propose that MLV PICs may either become specifically tethered to perichromosomal regions during mitosis, as has been described for certain pre-rRNA processing complexes (Dundr et al., 2000), or that they too may be transported into the nucleus through NPCs, but only for a very restricted period of the cell cycle that immediately follows mitosis. Because we postulate that the IN NLS of HIV-1 may have a role in directing the PIC to chromatin, we favor the notion that PICs are targeted to chromatin and that the loss of nuclear envelope integrity is necessary for MLV infection. Second, the requirement for PIC NLSs during all HIV-1 infections further suggests that transport through NPCs may be an essential postentry stage of the infection process. For instance, NPC binding and/or translocation may be necessary for aspects of uncoating or PIC maturation that must be executed for provirus formation to proceed. Notably, analogous steps have been described for infection by DNA viruses such as adenovirus where the viral capsids interact with NPCs and undergo substantial uncoating as a prerequisite to viral genome nuclear import (Greber et al., 1997).

Experimental Procedures

Molecular Clones

Carboxy-terminal fusions of IN to Myc-tagged PK were expressed using pcDNA1 or pcDNA1.1 vectors (Invitrogen) (Fouchier et al., 1997). All wild-type IN fragments were PCR amplified using the...
HIV-1 gp41 provirus expression vector pYU-2 as the template and inserted as EcoRI-XbaI PCR fragments. Alkaline-scaning mutagenesis of residues 161–173 or the aspartic acid at position 64 was performed using a subcloned SnaB1 to Ncol fragment (nucleotides 3111–5665); derivatives were then inserted as EcoRf-XbaI PCR fragments to create the PK-IN(1–288/full-length) or PK-IN(161–173) series of plasmids. Vectors expressing full-length IN with an amino-terminal FLAG tag or a carboxy-terminal fusion to a Vpr-RT chimera were derived from pFLAG-IN (Petit et al., 1999) or pLR2P-Vpr-RT-IN (Wu et al., 1997), respectively, by replacement of BamHf-XhoI PCR fragments. Proviruses carrying IN mutations between positions 161 and 173, or of D64, were generated by substitution of the SnaB1 to Ncol fragment. The vpr gene of pYU-2 (or mutated derivatives) was disrupted by fill-in of the Ncol site at position 5665 (Lupr constructs).

Cells and Cell Lines

The human cell lines HeLa, 293T, and CEM-SS, as well as modified versions of CEM-SS and C8166 expressing CCR5 (which allows infection of macrophages) and the HIV-1 pseudoviruses described above, were used. The polyHIV-1 cellular polyclonal population of CEM-SS/CCR5 that expresses ∼42,000 molecules of CCR5 per cell was selected by flow cytometry. The MAGI/RS and GHOST-X4/RS indicator cell lines have been described (Vodicka et al., 1997). The latter was obtained through the NIH AIDS Research and Reference Reagent Program (courtesy of Drs. Vineet KewalRamani and Dan Littman). Standard procedures were used for isolating peripheral blood mononuclear cells, monocyte-derived macrophages, and microglia (Fouchier et al., 1998).

Transfection of HeLa Cells, Indirect Immunofluorescence, and Western Blotting

HeLa monolayers were transiently transfected with the PK-IN, Flag-IN, or provirus expression vectors and subjected to indirect immunofluorescence using primary antibodies specific for M-Myc, Flag [monoclonal M2 (Sigma)] or IN [monoclonal (Fouchier et al., 1997)]. Following secondary hybridization with FITC-conjugated antibodies, samples were viewed using a Leica TCS 4D confocal microscope at ×400. Fusion protein expression was examined by Western blot analysis of cell lysates using Myc- or IN-specific antibodies, HRP-conjugated secondary antibodies, and ECL.

Virus Production and Protein Analysis

Wild-type and mutant stocks of HIV-1 were generated using 293T cells (Fouchier et al., 1997); for complementation studies, proviruses were cotransfected with Vpr-IN expression vectors at 2:1 ratio. Supernatant levels of p24Gag or reverse transcriptase (RT) were measured by ELISA or as enzymatic activity, respectively, and aliquots were stored at −80 °C. Stocks of MLV/V, a MLV-based retroviral vector encoding HIV-1 Tat, were quantitated by RT activity and stored at −80 °C (Bartz and Vodicka, 1997).

Virions pellets at 26,000 × g for 60 min (as well as corresponding cell lysates) were analyzed by Western blotting using antibodies specific for p24Gag (monoclonal), IN, or Vpr (rat polyclonal). Vpr immunization was evaluated by solubilization of virion pellets in the presence or absence of 5% β-mercaptoethanol, followed by Western blotting (Petit et al., 1999). Packaging of Vpr-IN fusion proteins was determined following virion production in the presence or absence of 1.25 μM saquinavir (an inhibitor of HIV-1 PR; Roche Pharmacueticals).

Purification and In Vitro Activity of Integrase

Hexahistidine-tagged IN proteins (residues 1–288) were expressed using pET28a (+) (Novagen) and the E. coli strain BL21(DE3). Purification by nickel-chelate affinity chromatography (Jenkins et al., 1996), and the eluted proteins were then stored at −1 mg/mL at −80 °C. IN core function in vitro was assessed using the reverse integration (or “disintegration”) reaction and annealed oligonucleotides, one of which was 5′ end labeled with 32P, that mimic the Y intermediate formed by the first cleavage-ligation reaction of integration (Chow et al., 1992).

Virus Replication and Measurement of Single-Cycle Infectivity

Spreading replica assays of HIV-1 were performed using the 293T-derived stocks and monitored as supernatant p24Gag ac-
cumulation. Single-cycle infectivity of HIV-1 was measured by challenging MAGI/CCR5 cells with serial dilutions of virus and staining for β-gal expression (Kimpton and Emerman, 1992); infectivity was defined as the number of β-gal-expressing cells divided by p24Gag input. To examine infectivities in nondividing cells, stocks corresponding to 15 ng p24Gag (wild-type IN) or 1.5 μg p24Gag (mutant IN) were used to challenge proliferating or cell cycle-arrested GHOST-X4/RS cells. Cells were arrested by irradiation with 3,500 rad from a 137Cs source, maintained in 5 μg/mL aphidicolin for 24 hr prior to infection, and checked for cell cycle status by propidium iodide staining and flow cytometry. As controls, cells were challenged with HIV-1 in the presence of the RT inhibitor AZT or with MLV/tat. The percentages of live cells that were GFP positive (i.e., those expressing Tat) were determined by flow cytometry; at least 10,000 events were counted for each sample.

Analysis of Reverse Transcription and Integration in Infected T Cells

2 × 106 CEM-SS/CCR5 cells (proliferating or arrested in G2) were challenged with wild-type, mutant, or complemented virus stocks corresponding to ~200 ng p24Gag by centrifugal inoculation (O’Do- nerty et al., 2000). Cells were washed with PBS, maintained in medium for up to 24 hr, and low and high molecular weight RNAs (which contain unintegrated or integrated viral RNAs, respectively) were extracted at various time points (Hirt, 1967). Cells were arrested either by incubation of cells in 1.4 μg/ml aphidicolin for 24 hr, washout and culture for 2 hr, irradiation with 800 rad from a 137Cs source, and maintenance for 18 hr in complete medium prior to challenge, or by irradiation alone. The presence of provirus DNA in Hirt pellets or whole-cell lysates was evaluated by Alu-PCR using the Alu-element primer 5′-GCCCT CCAAAAGTGGCCTGGATTACAG-3′ (Courcoul et al., 1996). Briefly, samples were phenol extracted, ethanol precipitated, resuspended, diluted in 10-fold increments, and subjected to nested PCR (the undiluted samples corresponded to the undiluted samples corresponded to 5 × 104 cells). Amplified products were analyzed by agarose gel electrophoresis and Southern hybridization using a random primed 32P-labeled LTR probe (EcoRf-Hael; nucleotides 35–637).

Hirt supernatant RNAs were examined for the presence of full-length linear and circular reverse transcripts by digestion with DpnII (to remove residual transfected RNAs) and BglII, resolution by agarose gel electrophoresis, and Southern hybridization using a 32P-labeled env probe (BglII-EcoRV; nucleotides 7722–9111). As shown in Figure 5B, linear DNA is detected as a 180-kb parent fragment, and the 1- and 2-LTR circular RNAs are detected as 2368- and 3198-base pair fragments, respectively.

Subcellular Fractionation and Analysis of Postentry Localization of Integrase

For each virus/time point sample, 105 cells were spin infected with stocks corresponding to ∼300 ng p24Gag for 60 min. After washing, the cultures were maintained for up to 6 hr. Cells collected at various times, swelled for 10 min on ice in STM buffer (250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 1 mM MgOAc2), lysed by Dounce homogenization, and centrifuged at 2000 × g for 10 min. Supernatant (cytoplasmic) samples were separated into soluble and insoluble fractions by centrifugation at 436,000 × g for 30 min and then adjusted to, or resuspended in, 1 × RIPA, respectively. In some cases, total proteins were then precipitated with acetone prior to further analysis (see below). The pellets (which mostly represent nuclei and associated structures) were resuspended in 100 μg/mL digitonin buffered with 20 mM HEPES, 110 mM KOAc, 3 mM MgOAc2, incubated at 25°C for 15 min, and centrifuged at 2000 × g for 10 min. The pellets were resuspended in STM, adjusted to 1 × RIPA, and sheared using a 26-gauge needle. After centrifugation at 25,000 × g for 30 min, the supernatants (soluble nuclear fractions) were removed and in some cases acetone precipitated, and the insoluble pellets were resuspended in sample buffer.

Proteins of interest were either analyzed by immunoprecipitation followed by Western blotting (cytoplasmic or soluble nuclear sam-
plies in RIPA) or by Western blotting alone (acetone precipitates or pelleted insoluble material) using antibodies specific for IN (monoclonal and rabbit polyclonal), LDM (polyclonal raised in sheep [Cortex Biochemicals]), hnRNP C proteins [monoclonal], or α-tubulin [monoclonal (Molecular Probes)].

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