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HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway

(nuclear import/human immunodeficiency virus)

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ABSTRACT **The karyophilic properties of the HIV-1 nucleoprotein complex facilitate infection of nondividing cells such as macrophages and quiescent T lymphocytes, and allow the *in vivo* delivery of transgenes by HIV-derived retroviral vectors into terminally differentiated cells such as neurons. Although the viral matrix (MA) and Vpr proteins have previously been shown to play important roles in this process, we demonstrate here that integrase, the enzyme responsible for mediating the integration of the viral genome in the host cell chromosome, can suffice to connect the HIV-1 preintegration complex with the cell nuclear import machinery. This novel function of integrase reflects the recognition of an atypical bipartite nuclear localization signal by the importin/karyopherin pathway.**

Terminally differentiated macrophages, mucosal dendritic cells and quiescent T lymphocytes are nonproliferating targets of critical importance for the transmission and the spread of HIV-1. HIV can infect nondividing cells because its preintegration complex is recognized by the cell nuclear import machinery and actively transported through the nucleopore (1–3). Correspondingly, HIV-derived vectors can transduce growth-arrested and terminally differentiated cells *in vitro*, and allow for the efficient *in vivo* delivery, integration, and long-term expression of transgenes into nonmitotic targets such as adult neurons (4, 5). In contrast, oncoretroviruses such as the murine leukemia virus and oncoretroviral vectors cannot traverse an intact nuclear envelope, precluding integration in the absence of mitosis (6–9).

The karyophilic properties of the uncoated HIV-1 preintegration complex reflect the presence of nuclear localization signals (NLS) on at least two of its components, matrix (MA) and Vpr (10–12). MA contains in its proximal portion a stretch of basic residues that acts as an NLS recognized by the importin/karyopherin pathway (3, 10). In the absence of a functional *vpr* gene, MA NLS mutant viruses fail to replicate efficiently in macrophages (10, 12) and cannot establish a stable infection intermediate in quiescent T lymphocytes (12). The Vpr nuclear localization motif is not precisely delineated, and the nature of its cellular receptor remains to be defined. However, Vpr can rescue the ability of MA-mutated viruses to infect terminally differentiated macrophages (11).

In addition to these two viral proteins, a third component of the HIV-1 nucleoprotein complex, integrase (IN), participates in its transport to the nucleus. IN mediates the integration of the viral DNA into the host cell chromosome, and is closely associated with the viral genome. During virion maturation, IN binds the tyrosine-phosphorylated C terminus of a subset of MA proteins, thereby recruiting these molecules into the virion core and subsequently into the uncoated viral nucleoprotein complex (13). In the absence of IN, or when the C-terminal tyrosine of MA is replaced by a phenylalanine, the HIV-1 nucleoprotein complex lacks the karyophilic potential of MA (13, 14).

Here, we add a new dimension to the understanding of HIV-1 nuclear import by implicating the NLS-mediated recognition of IN by members of the importin/karyopherin-α family as another key to this process. The outcome of this association is to facilitate infection of nondividing cells both *in vitro* and *in vivo*.

MATERIALS AND METHODS

DNA Constructions. Plasmid R7 contains HIV-1_{HXB2} proviral DNA with a full-length *nef* reading frame (15) and expresses a nonfunctional, truncated Vpr protein. In R9, previously called R8 (14), the *Bss*HII–*Bam*HI fragment of R7, extending from U3 to the 3′ portion of *env*, is replaced with a corresponding fragment from HIV-1_{NL4-3} proviral DNA (16). The ΔIN mutant was previously described (13). The ΔVpr version of R9 was generated by introducing a 4-nucleotide insertion in *vpr* by digestion with *Eco*RI followed by Klenow treatment. The macrophage-tropic variant R7.Bal and its derivatives were previously described (12, 14). An IN expression vector was obtained by fusing the *gag* AUG with the first codon of IN in the R7 proviral construct, using PCR. Sequences encoding the HIV-1 MA, IN, Nef, and CA proteins were cloned into pGEX-2T (Pharmacia) to produce glutathione *S*-transferase (GST) fusion proteins. PCR-mediated mutagenesis was used to generate the IN and MA NLS mutants in the various vectors. Vectors encoding a myc-tagged form of human karyopherin-α2 or Rch1 (17) and recombinant forms of karyopherin-α1 and of karyopherin-β (18) were kind gifts from M. Oettinger and G. Blobel, respectively. The karyopherin-α2 variant used in these experiments lacks 32 N-terminal residues (17).

Cell Lines, Transfections, and Infections. Cell culture, transfections, viral production as well as nucleoprotein complex purification and subcellular localization studies were performed as previously described (12–14, 19). For single-round infectivity measurements, 8×10^4 dividing or 1.5×10^5 γ-irradiated (5,000 rads) P4 cells (20) were inoculated with viral stocks containing 1 ng of p24 antigen, and cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) after 48 h. To block nuclear import in P4 cells, HPLC-purified peptides corresponding to the prototypic SV40 T antigen NLS in the sense (PKKKRKVEDPYC) and reverse (PDEVKRKKKPYC) orientations were used as described (3).

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Abbreviations: NLS, nuclear localization signal(s); MA, matrix; IN, integrase; GST, glutathione *S*-transferase; RT, reverse transcriptase; NC, nucleocapsid; CA, capsid.

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Detection of Viral Proteins. HIV-1 p24 antigen values in virus and cell extracts were determined by ELISA (DuPont). Reverse transcriptase (RT) activity was monitored by a so-called exogenous RT assay (21) with minor modifications (22); nuclear extracts were dialysed before performing RT assays. Other viral proteins were detected by Western blot analysis with specific antibodies with or without prior immunoprecipitation using antibodies covalently bound to beads, as described (14). Polyclonal sera against HIV-1 MA and IN were obtained by immunizing rabbits with *Escherichia coli*-produced recombinant proteins (13). Rabbit anti-nucleocapsid (NC) serum was a gift of L. Henderson (National Cancer Institute–Frederick Cancer Research and Development Center). Rabbit sera reacting against importin/karyopherin-α and -β were a kind gifts from M. Oettinger and S. Adam, respectively (17, 23).

Subcellular Localization Studies. For studying the subcellular localization by microinjection, purified GST fusion proteins were labeled with a 25 molar excess of fluorescein isothiocyanate–maleimide (Pierce) in PBS (pH 7.4), 25% *N*,*N*-dimethylformamide for 2–3 h on ice. The resulting labeled protein was then separated from unincorporated label by G-50 Sephadex (equilibrated with PBS, pH 7.4) chromatography followed by concentration by centrifugation in a Centricon C-10 unit. Two days before injection, a confluent flask of COS-7 cells grown in DMEM supplemented with 10% fetal calf serum was trypsinized, diluted 1:6 or 1:12, and seeded onto 25-mm-diameter glass coverslips. For microinjection, the cells were mounted into a custom temperature-controlled chamber and placed atop an inverted microscope equipped with microinjection and video monitoring equipment and a confocal scanning laser module (24). Approximately 50 fl of labeled protein mixed with Cy3-labeled IgG were injected into the cytoplasm of COS-7 cells incubated at 20°C in CO_2-deficient medium (GIBCO). Import was observed in 1–2 min. Conservative operating conditions of the confocal microscope (low laser illumination and moderate to high photomultiplier gain) were employed to minimize photobleaching resulting from the laser illumination at two or three time points.

Protein Interaction Assay. HIV-1 IN, CA, Nef, and MA were expressed as GST fusion proteins (Pharmacia) in the *E. coli* strain DG98, whereas yeast karyopherin-α and -β in the protease-deficient *E. coli* strain BLR (Novagen) were purified from *E. coli* lysates on glutathione-agarose beads as described (18). Interactions between GST–HIV-1 fusion proteins and human Rch1 and between GST–IN and recombinant forms of karyopherins were performed as described (3, 18).

RESULTS

Evidence for a Third Mediator of HIV-1 Nuclear Import. Three lines of evidence suggested that MA and Vpr are not the sole viral proteins responsible for allowing HIV-1 infection of nondividing cells. First, in terminally differentiated macrophages the defective phenotype of HIV-1 variants mutated in Vpr and in either the NLS or the C-terminal tyrosine of MA was abrogated at a high multiplicity of infection (moi). In a representative experiment (Fig. 1), while the growth of these mutated viruses was severely impaired at a low dose inoculum [i.e., 0.2 (data not shown) or 2 ng p24 antigen for 2×10^5 cells], their defective phenotype was abrogated when a higher amount of virus was used (i.e., 40 ng of p24 antigen). In both settings, p24 antigen levels remained low when the cells were maintained in the presence of 3′-azido-3-deoxythymidine (AZT), indicating that the observed virus production resulted from a bona fide infection, rather than from the regurgitation of internalized virions by the cells. A further and stronger proof of the existence of an additional mediator of HIV-1 nuclear import was provided by results obtained through single-round infectivity assays in P4 cells, which are $CD4^+$ HeLa cells containing a HIV LTR-driven β-galactosidase reporter gene for easy scoring of infection (20). A dually mutated ($MA_{\Delta NLS}\Delta Vpr$) HIV-1 variant was as infectious as wild-type and singly mutated (ΔVpr or $MA_{\Delta NLS}$) viruses in γ-irradiated P4 cells, in contrast with murine leukemia virus that was inactive in this context (Table 1). Finally, our suspicion of a third mediator of HIV-1 nuclear import was reinforced by studies performed with an HIV-derived retroviral vector, where a Vpr-, MA NLS-defective vector could mediate the transfer of a β-galactosidase reporter gene into adult rat neurons, albeit less efficiently than its wild-type counterpart (25).

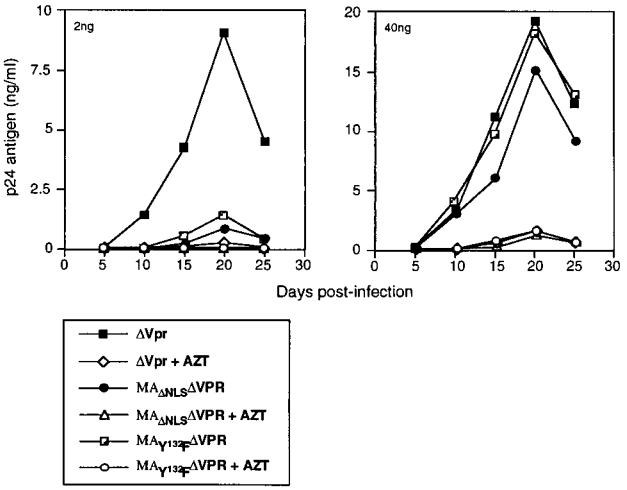

FIG. 1. The growth defect of MA/Vpr double mutant HIV-1 in terminally differentiated macrophages is masked at a high moi. Monocyte-derived macrophages were cultivated for 3 weeks in the absence of growth factors. Cells (5×10^5) plated in 6-well dishes were infected with 2 (*Left*) or 40 (*Right*) ng p24 of the indicated viruses. ΔVpr: R7.BaL, a macrophage tropic HIV-1 strain encoding a truncated, functionally defective Vpr protein; $MA_{\Delta NLS}\Delta Vpr$ and $MA_{Y^{132}F}\Delta Vpr$: R7.BaL derivatives, with $KK^{27} \rightarrow TT$ and $Y^{132} \rightarrow F$ changes in MA, respectively. After washing off unadsorbed virus, cells were incubated in 3 ml of medium with 10 μM 3′-azido-3-deoxythymidine (AZT) where indicated, and p24 antigen production was monitored by ELISA. These growth curves are representative of seven independent experiments.

Third Mediator of HIV-1 Nuclear Import Acts in an NLS-Dependent Manner. These data pointed to an activity that could substitute for MA and Vpr in facilitating the nuclear transport of the HIV-1 preintegration complex. To explore this issue further, dividing or growth-arrested P4 cells were exposed to wild-type or mutated HIV-1 derivatives in the presence of peptide corresponding to the prototypic SV40 large T antigen NLS, or to reverse peptide as control (3). NLS

Table 1. Relative infectivity of HIV-1 derivatives in growth-arrested HeLa cells

Viruses	Without treatment	Plus NLS peptide	Plus reverse peptide
WT	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.06
MA ΔNLS	0.96 ± 0.06	0.93 ± 0.05	1.04 ± 0.05
ΔVpr	1.04 ± 0.07	0.18 ± 0.01	1.00 ± 0.06
MA ΔNLS ΔVpr	1.04 ± 0.06	0.18 ± 0.01	1.04 ± 0.05
MLV	0.09 ± 0.001	0.09 ± 0.001	0.09 ± 0.001

Growing and γ-irradiated P4 cells were infected with 1 ng of p24 antigen of the indicated viruses, either without treatment or in the presence of NLS peptide in the sense or reverse orientations, as indicated. After 48 h, infectivity was scored by X-Gal staining. In a typical experiment, the efficiency of the wild-type virus in arrested cells was 23–28% of that observed in growing cells. Results are expressed as a function of the infectivity of the wild-type virus in arrested cells, itself arbitrarily given a value of 1.00 ± SD of replicate determinations.

peptide had no effect in dividing cells (data not shown) and did not affect the titer of the wild-type virus in γ-irradiated cells (Table 1). In contrast, it strongly blocked infection of nondividing HeLa cells by *vpr*-defective strains (ΔVpr or $MA_{\Delta NLS}$ΔVpr) (Table 1). This result confirmed that Vpr governs HIV-1 nuclear import in a manner distinguishable from that determined by MA, as previously reported (3). It also indicated that the third karyophilic determinant of HIV-1 acts most likely via the recognition of a prototypic NLS by the importin/karyopherin pathway. This data was also consistent with our previous observation that the transduction of primary macrophages by an HIV-derived vector is more efficiently blocked by addition of NLS peptide than by a double MA NLS/Vpr mutation (4).

IN Can Mediate HIV-1 Nuclear Import. In addition to the karyophilic signals present at the proximal end of MA and in Vpr, putative NLS are found in the C-terminal portion of MA ($KKKK^{116}$), between the two zinc finger domains of NC ($KKKK^{37}$) and in IN ($KELKK^{160}$, KRK^{188}, $KELQKQITK^{219}$), $PRRKAK^{265}$) (16). Mutating the putative NLS at the distal end of MA and in NC, in the context of a virus defective for both Vpr and the N-terminal MA NLS, did not affect HIV-1 infection of γ-irradiated P4 cells (data not shown). Growth-arrested P4 cells were then infected with an HIV-1 derivative that lacked IN (ΔIN). At 1 and 8 h postinfection, cytoplasmic and nuclear extracts were analyzed for the presence of NC and RT, two components of the preintegration complex (13) (Fig. 2). Of note, MA could not be used for this analysis, as we previously demonstrated that its incorporation into the preintegration complex requires the presence of IN (13). In wild-type infected cells, NC and RT were detected only in the cytoplasm after 1 h but had partly migrated to the nucleus after 8 h, whereas capsid (CA) remained associated with the cytoplasm as previously described (13, 14). Similar results were observed with HIV-1 mutants defective either for Vpr and the MA NLS ($MA_{\Delta NLS}$Δvpr) or for IN (ΔIN). In contrast, no nuclear translocation of NC and RT was observed in the case of a triply mutated derivative that lacked the MA NLS, Vpr, and IN ($MA_{\Delta NLS}$ΔvprΔIN), strongly suggesting that IN is a mediator of HIV-1 nuclear import.

IN Recognizes Karyopherin-α via a Bipartite NLS. To confirm that IN is a karyophilic protein, a fluorescein-conjugated recombinant molecule in which IN was fused at the C terminus of GST (GST–IN) was injected into the cytoplasm of COS cells, and its subcellular localization was followed by confocal microscopy (Fig. 3). Microinjections of BSA, GST, and GST fused to wild-type or NLS-mutated versions of MA (GST–MA and GST–$MA_{\Delta NLS}$, respectively) served as controls. Like BSA, GST–$MA_{\Delta NLS}$ localized to the cytoplasm, as did GST (data not shown). By contrast, in a temperature-dependent manner, GST–MA was found in abundance in the nucleus, and GST–IN was detected almost exclusively in this compartment.

The results obtained with NLS peptide (Table 1) suggested that the karyophilic properties of IN reflect its ability to interact with the karyopherin pathway. Consistent with this model, karyopherin-α (17) overexpressed in 293 cells bound GST–IN, but not GST–CA, or GST–Nef (Fig. 4*A*). NLS peptide, but not reverse peptide, blocked this interaction (Fig. 4*B*). In an attempt to mimic

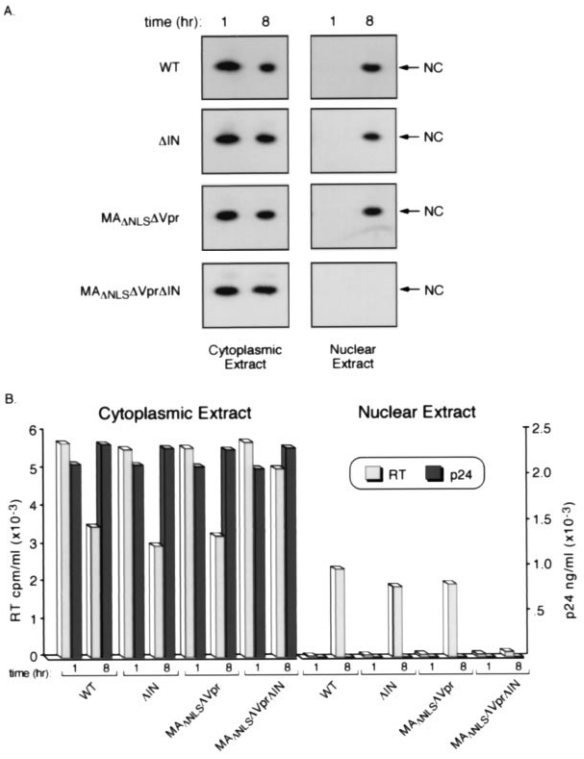

FIG. 2. Subcellular localization of viral proteins during the early steps of infection. P4 cells growth-arrested by γ-irradiation were acutely infected with wild-type HIV-1 (produced by transfection of 293 cells with R9 plasmid), or with derivatives lacking IN (ΔIN) and/or containing mutations in the MA NLS and Vpr ($MA_{\Delta NLS}$ΔVprΔIN and $MA_{\Delta NLS}$ΔVpr, respectively). Cells were fractionated at 1 and 8 h postinfection in cytoplasmic and nuclear extracts. The presence of various viral proteins was monitored by immunoprecipitation followed by Western blot analysis for NC (*Upper*), or by measuring RT activity and p24 CA antigen content (*Lower*).

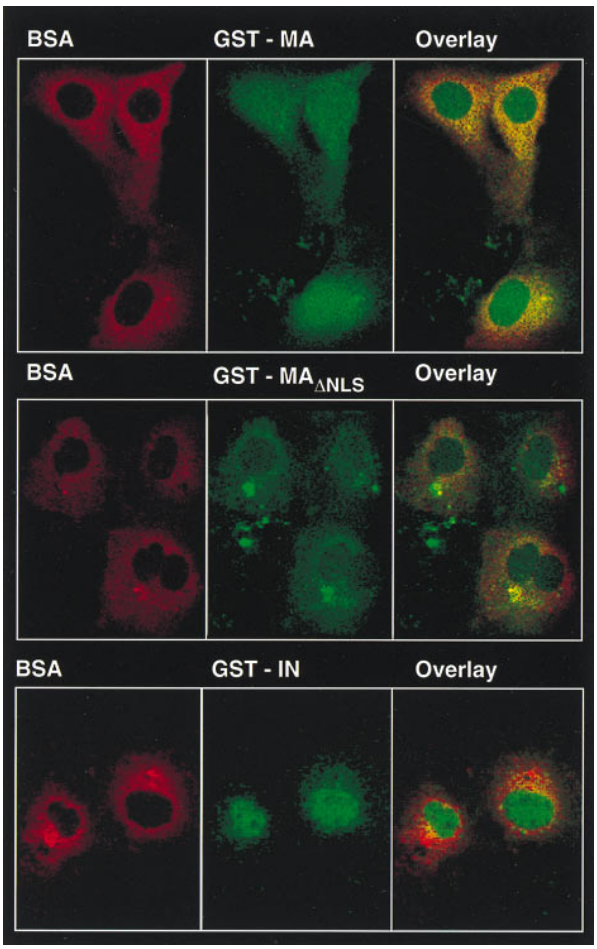

FIG. 3. IN is a karyophilic protein. Fluorescein-conjugated versions of the indicated recombinant GST derivatives and rhodamine-conjugated BSA were injected in the cytoplasm of COS cells. Analysis was performed by confocal microscopy.

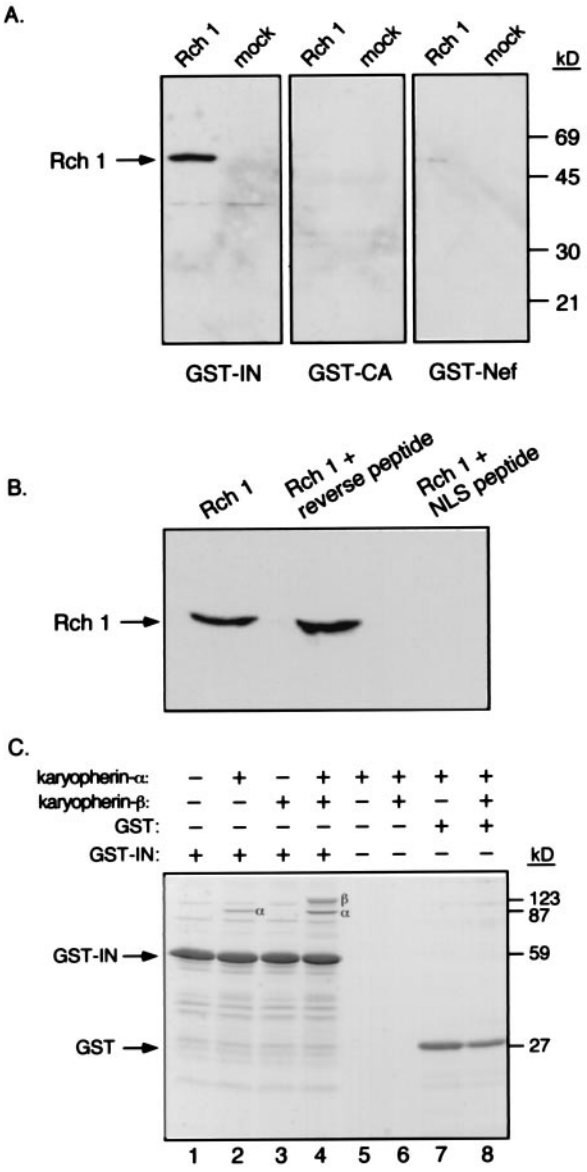

FIG. 4. NLS-dependent binding of IN to importin/karyopherin-α. (*A*) Cytoplasmic extracts from 293 cells expressing a *myc*-tagged form of Rch1 were incubated with a GST–IN, GST–CA or GST–Nef fusion proteins. Bound material was analyzed by Western blot with anti-*myc* antibody. (*B*) Same experiment, except for the preincubation of the cytoplasmic extract with peptide corresponding to the large-T-antigen NLS in the sense (PKKKRKVEDPYC) or reverse (PDEVKRKKKPYC) orientations. (*C*) Recombinant forms of the indicated proteins were incubated *in vitro*, and complexes were recovered by affinity purification with agarose beads. Bound material was analyzed by SDS/PAGE and Coomassie blue staining.

the events presumably responsible for docking IN at the nucleopore, recombinant forms of yeast karyopherin-α and karyopherin-β (18) were successively added to GST–IN (Fig. 4*C*). IN could associate with karyopherin-α, whereas karyopherin-β bound to GST–IN/karyopherin-α complexes but not to GST–IN alone.

Deletion analyses indicated that the C-terminal region of IN, downstream of amino acid 185, is involved in binding importin/karyopherin-α (Fig. 5*A*). Two potential NLS in this region, one proximal (NLS_P) around positions 186–189 (KRK^{188}) and one distal (NLS_D) encompassing residues 211–219 ($KELQKQITK^{219}$), were mutated within the context of GST–IN. Single NLS_P or NLS_D IN mutants had a severely decreased affinity for karyopherin-α, whereas the double mutant was completely defective in this assay. NLS_P or NLS_D thus appear constitute a bipartite NLS rather than two discrete motifs. Confirming the functional significance of the IN–karyopherin interaction, wild-type IN was found both in the cytoplasm and in the nucleus, whereas NLS-mutated IN was restricted to the cytoplasm (Fig. 5*B*). Also, fluorescein isothiocyanate-labeled GST–$IN_{\Delta NLSP}$ and GST–$IN_{\Delta NLSD}$ failed to localize to the nucleus following microinjection into the cytoplasm of COS cells (not illustrated).

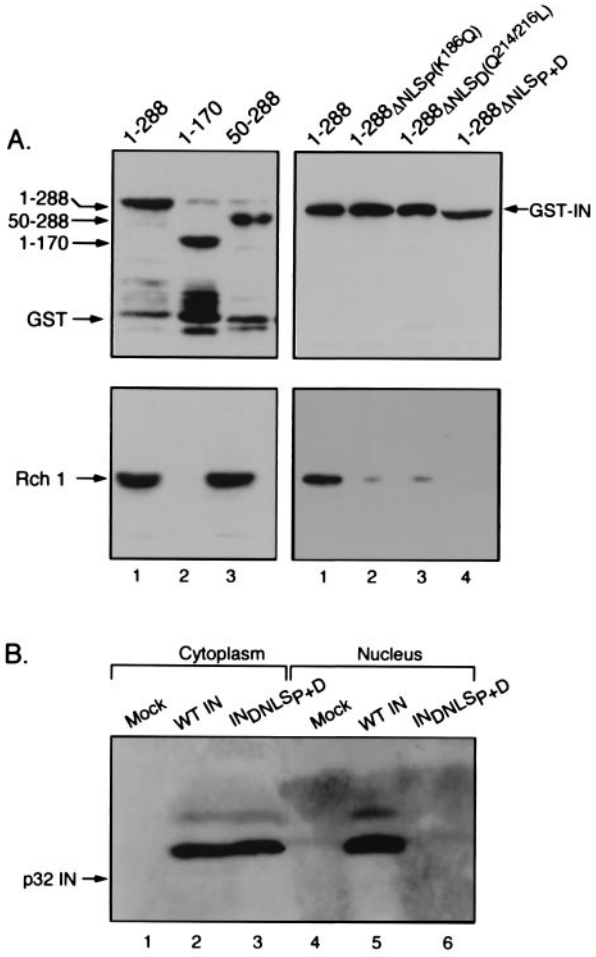

FIG. 5. A bipartite NLS in the C-terminal portion of IN. (*A*) Same experiment as in Fig. 4*A*, with GST–IN derivatives comprising either truncated (*Left*) or mutated (*Right*) forms of IN. Analysis of bound material was performed by Western blot using GST- (*Upper*) and *myc*- (*Lower*) specific antibodies. Numbers on top of lanes correspond to amino acids of IN present in the fusion protein; 1–288 represents a full-length IN protein. (*B*) 293 cells expressing wild-type or NLS-mutated versions of IN were fractionated in cytoplasmic and nuclear extracts. The subcellular localization of IN was analyzed by Western blot. $IN_{\Delta NLSP+D}$ contains mutations in both NLS_P and NLS_D ($K^{186}Q$ and $Q^{214/216}L$, respectively).

NLS-Dependent Recruitment of IN by Karyopherin Facilitates HIV-1 Nuclear Import. Karyopherin-α could be coimmunoprecipitated with IN in uncoated viral nucleoprotein complexes purified from the cytoplasm of freshly infected T lymphoid cells (Fig. 6*A*). Noteworthy, the NLS receptor bound more tightly to IN than to MA, as revealed by comparing the detergent sensitivities of the corresponding complexes. This difference may reflect the presence of a bipartite NLS in IN, whereas a simpler motif is found in MA; also, it correlates with the more efficient nuclear migration of GST–IN compared with that of GST–MA (Fig. 3). Finally, the nuclear transport of the HIV-1 preintegration complex in growth-arrested HeLa cells was profoundly inhibited when the IN NLS was inactivated in the context of an MA NLS/Vpr-defective HIV-1

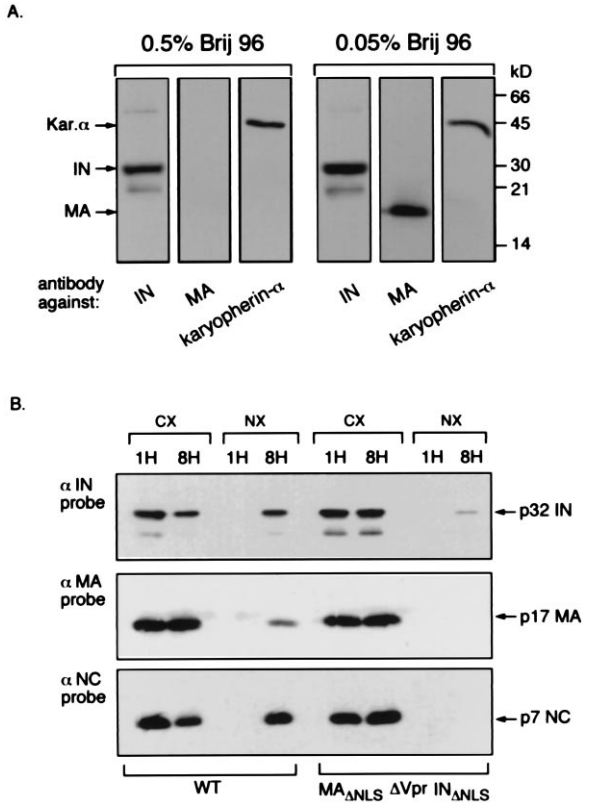

FIG. 6. The IN–karyopherin interaction can mediate HIV-1 nuclear import. (*A*) Preintegration complexes purified from the cytoplasm of acutely infected SupT1 cells were analyzed by immunoprecipitation with a mixture of antibodies against karyopherin-α and karyopherin-β, followed by Western blot analysis with antibodies against IN, MA, or karyopherin-α, in the presence of the indicated amount of detergent. (*B*) Same experiment as in Fig. 2, in γ-irradiated P4 cells acutely infected with wild-type (WT) or triply mutated ($MA_{\Delta NLS}\Delta VprIN_{\Delta NLS}$) HIV-1. Cytoplasmic (CX) and nuclear (NX) fractions were analyzed by immunoprecipitation and Western blot with the indicated antibodies. The IN mutation targeted both NLS_P and NLS_D, as described for Fig. 5. RT exhibited a subcellular localization similar to that of NC, as in Fig. 2 (not shown).

variant, as assessed by monitoring the subcellular localization of IN, MA, and NC in γ-irradiated P4 cells freshly infected with the triply mutated virus (Fig. 6*B*). Of note, virions and preintegration complexes of this virus presented densities and levels of RT activity that were identical to those of wild type (data not shown). Altogether, these results indicate that the recognition of IN by the karyopherin pathway can mediate HIV-1 nuclear import, thereby facilitating infection of nondividing cells.

DISCUSSION

This study reveals a novel function for a retroviral IN, by demonstrating that HIV-1 IN can govern the nuclear transport of the viral preintegration complex. The enzyme thus promotes HIV-1 infection of nondividing cells by a dual mechanism, as it first recruits the karyophilic potential of MA into the viral nucleoprotein complex and then is itself recognized by the cell nuclear import machinery. By analogy, it will be interesting to explore the possibility that IN might fulfill a similar function for other lentiviruses, most of which lack a Vpr protein yet can infect nondividing cells. Quite remarkably, the IN protein of the yeast retrotransposon Ty1 was just discovered to contain an NLS, the mutation of which abolishes the nuclear localization of Ty1 IN and inactivates transpositional integration (M. A. Kenna, C. B. Brachmann, S. E. Devine, and J. D. Boeke, personal communication; S. P. Moore, L. A. Rinekel, and D. J. Garfinkel, personal communication).

The suggestion that the MA NLS and Vpr were not the sole viral factors responsible for HIV-1 nuclear import came in part from the observation that, in terminally differentiated macrophages, the replicative capacity of an HIV-1 strain altered in both of these motifs was restored by increasing the moi. One possible explanation for this phenomenon is that only a small fraction of HIV-1 particles might be competent for infection of macrophages through an MA/Vpr-independent mechanism, so that this subset will be present only in high moi. inoculums. This moi effect might explain why Freed *et al.* (28–30) have failed to observe an influence of MA and Vpr on the efficiency of HIV-1 replication in macrophages, in contrast with the findings of three other groups including ours (3, 10, 14, 31). In growth-arrested $CD4^+$ HeLa cells, an MA NLS–Vpr dually defective HIV-1 derivative was normally infectious, as revealed in a single-round infectivity assay. This corroborates our recent finding that a similarly mutated HIV-derived vector could transduce nonmitotic cells *in vitro* and could deliver transgenes into terminally differentiated neurons *in vivo*, albeit at a reduced efficiency compared with wild type (25).

The finding that IN can be a direct mediator of HIV nuclear import does not invalidate our previous conclusions on the roles that MA and its binding of IN play in this process. We previously demonstrated that MA can independently recognize karyopherin-α, yet that it is recruited in the preintegration complex only if it is tyrosine phosphorylated and if IN is present (3, 13, 14). The present data add another dimension by revealing that IN is itself recognized by karyopherin-α. Of note, injection studies cannot address the role of MA phosphorylation and of the MA–IN interaction because the recombinant protein that is used is not phosphorylated.

The karyophilic properties of IN were demonstrated within the context of GST fusion proteins, as well as by mutations inactivating the IN NLS, and corroborated the NLS-mediated recognition of IN by karyopherin-α. This contradicts a recent study which concluded that HIV-1 IN is not a karyophile (32). However, this other study was performed within the sole context of a fusion protein in which β-galactosidase was placed at the C terminus of IN. It is likely that, in this configuration, the IN NLS was masked. Compared with the prototypic bipartite signal found in nucleoplasmin, in which a spacer of only 10 amino acids is present (reviewed in ref. 33), the NLS of IN is atypical since NLS_D is located 22 amino acids downstream of NLS_P. Remarkably, the organization of the Ty1 IN NLS is very reminiscent, as it consists in two KKR sequences separated by 29 amino acids (ref. 18; Kenna *et al.*, personal communication). According to the crystal structure of the HIV-1 IN core domain, NLS_P forms an exposed loop which is flanked by two α-helices (34). Unfortunately, the structure of the C-terminal part of IN, in which NLS_D resides, is not known, so that the topology of the entire signal cannot yet be reconstructed.

Functionally, the residues involved in connecting IN with the importin/karyopherin pathway must participate in additional activities of the protein. Indeed, HIV-1 strains mutated in either NLS_P or NLS_D are replication defective even in the presence of functional MA and Vpr proteins, irrespective of the proliferative status of the target cells (data not shown). This phenotype reflects a block in integration, while all other aspects of viral replication, including reverse transcription and nuclear import, proceed normally (data not shown). A similar finding was recently reported by other investigators, who further claimed that mutations in NLS_P or NLS_D did not affect the *in vitro* enzymatic activity of IN (35). A similar finding was recently reported by other investigators, who further described that mutations in NLS_P or NLS_D did not affect the *in vitro* enzymatic activity of IN (35). One interesting possibility is that this region of the protein might facilitate the intranuclear

trafficking of the preintegration complex and its docking on chromosomes. In that respect, IN of avian sarcoma virus was recently demonstrated to carry an NLS, even though this virus cannot infect nondividing cells (32). This lends some support to a model in which the IN NLS helps the preintegration complex gain access to chromatin even during mitosis. Alternatively, the residues involved in making the HIV-1 IN NLS could participate in an enzymatic step of the integration process which is not accurately reflected by the *in vitro* assay. Of note, results obtained in the yeast two-hybrid system suggest that mutations in NLS_P and NLS_D interfere with IN dimerization (unpublished data).

The present work increases to three the number of viral components that can govern the nuclear transport of the HIV-1 nucleoprotein complex. This apparent plethora of mediators is not unprecedented. Nuclear localization sequences have been identified on nucleoprotein as well as on all three subunits of the RNA polymerase complex of influenza virus (reviewed in ref. 26). Similarly, the NLS-bearing VP1, VP2, and VP3 proteins are putative mediators of simian virus 40 nuclear import, whereas hexon and several of the coat proteins are candidates for this function in adenovirus (reviewed in ref. 27). At least in the case of HIV-1, it is likely that redundancy was selected to ensure the completion of a step critical for viral replication irrespective of the cellular environment. In support of this model, the relative contribution of MA, IN, or Vpr to HIV-1 nuclear import depends on the cellular context. For instance, IN is sufficient to mediate HIV-1 nuclear migration in monocyte-derived macrophages inoculated with high doses of virus and in some epithelial or fibroblastic cell lines (this work), as well as *in vivo* in neurons (25). In contrast, the influence of MA and Vpr predominates in macrophages exposed to a low moi and in quiescent peripheral blood T lymphocytes, where a virus mutated in both of these determinants is markedly defective (3, 10, 12, 14). Under some circumstances, the various mediators of HIV-1 nuclear import may exert additive effects. This is the case of MA and Vpr in monocyte-derived macrophages, both for HIV-1 (3, 11, 30) and for HIV-derived vectors (4). At least two factors could account for the relative cell-specificity of the requirements of HIV-1 nuclear import. First, putative high affinity receptors for one or the other of the three mediators of this process might be differentially expressed. Second, the modalities of virus uncoating might vary from cell type to cell type, influencing the degree to which a given NLS is exposed in the resulting preintegration complex.

A significant effort is deployed to identify inhibitors of the HIV-1 IN. While current approaches screen candidate compounds solely for their ability to block the enzymatic activity of the protein, it might be judicious to examine as well their potential effect on the binding of IN to matrix and karyopherin-α. New avenues for the development of antiviral drugs may be revealed.

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