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Reduced Nuclear Import of Human Immunodeficiency Virus Type 1 Preintegration Complexes in the Presence of a Prototypic Nuclear Targeting Signal

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Nuclear import of the retroviral preintegration complex and integration of retroviral with host cell DNA are essential steps for completion of the virus life cycle. The preintegration complex of the lentivirus human immunodeficiency virus type 1 (HIV-1) displays karyophilic properties and, as a consequence, is rapidly directed to the host cell nucleus by an energy-dependent transport pathway. The karyophilic properties of nuclear proteins are governed by a nuclear localization sequence, the targeting function of which can be inhibited in the presence of excess targeting signals. Here we present evidence that the nuclear import of a large karyophile—the preintegration complex of HIV-1—is inhibited in the presence of a prototypic nuclear targeting signal of simian virus 40 T antigen. This points to a novel strategy which prevents establishment of the provirus by interrupting nuclear localization of HIV-1 DNA.

Permissiveness of the host cell to productive infection by most oncoretroviruses is cell cycle dependent (26). Thus, in nondividing cells, virus replication is arrested at a stage between retroviral DNA synthesis and integration of viral with host cell DNA (21). In contrast, establishment of the integrated provirus following infection by lentiviruses, such as human immunodeficiency virus type 1 (HIV-1), is independent of host cell division and is reflected by virus replication in terminally differentiated and nondividing cells such as those of monocyte-macrophage lineage (27) or in G_2- and S-phase-arrested $CD4^+$ cells (4, 14). This pattern of HIV replication can be accounted for in part by the karyophilic properties of the viral preintegration complex (4). This complex, which contains all the necessary functions for reverse transcription of viral RNA and integration of viral cDNA with host cell DNA, rapidly undergoes nuclear import by an energy-dependent process which is independent of host cell division. Determinants within the preintegration complex of HIV-1 which impart this karyophilic property are contained within the viral core (3, 14, 15). Thus, murine leukemia virus pseudotypes, possessing *gag-pol* proteins of HIV-1, will replicate in nondividing cells.

Nuclear localization signals (NLSs) which govern the karyophilic properties of nuclear proteins have been characterized for a wide variety of viral and cellular proteins. Although there is no clear concensus, NLS motifs generally resemble either the single basic domain simian virus 40 (SV40) large T antigen sequence (PKKKRKV) or the double basic domain nucleoplasmin sequence (KRPAATKKAGQAKKK) (6). In addition, the nuclear targeting properties of diverse NLS motifs both in peptide form (10, 17) and in the context of the native protein can be inhibited in the presence of excess T antigen NLS peptide analogs. This suggests that the transport pathway is saturable and that the NLS receptor, which transports karyophiles to the nuclear pore, displays a ubiquitous recognition for diverse NLS motifs. In this study, we have examined the influence of the prototypic SV40 T antigen NLS on the nuclear import properties of a large karyophile, namely, the preintegration complex of HIV-1.

Identification of cytoplasmic and nuclear forms of viral cDNA. The synthesis of two distinct molecular forms of HIV-1 DNA as used to monitor de novo synthesis of HIV-1 DNA following acute infection and transport of nascent viral cDNA to the host cell nucleus. Primers spanning *pol* sequences in the HIV-1 genome (4, 5) were used to amplify intermediate and late products of reverse transcription (11). This approach, which excluded early reverse transcription products from the analysis, was necessary since such products do not represent de novo synthesis of viral DNA but rather reverse transcription products of the virion (16, 25). Transport of nascent viral cDNAs from cytoplasmic to nuclear compartments of the cell was monitored by using PCR and primers which span the junction between the 5′ long terminal repeat (LTR) R region and 3′ LTR U5 region as created during formation of the 2 LTR circle. Although such circle forms of viral DNA do not appear to be integration precursors (2, 15), they are formed only after synthesis of linear viral DNA and its transport to the nucleus (4, 5, 26) and thus represent a marker for nuclear localization of viral DNA. Cells were arrested at the G_1-S interface of the cell cycle by the action of aphidicolin (a reversible inhibitor of eukaryotic DNA polymerase α) as detailed elsewhere (4). Aphidicolin was used as an inhibitor of cell proliferation since it inhibits DNA synthesis but has no effect on early events in the life cycle of HIV-1 leading up to integration of viral with host cell DNA in the nucleus (4). Under conditions of G_1-S-phase arrest, preintegration complexes of HIV-1 are imported to the nucleus by active transport, while diffusion processes drive nuclear import of these complexes in proliferatintg cells (4). Metabolic blocks which arrest cells in G_0 (sodium azide and *d*-D-glucose, or serum starvation) were not used since preintegration complexes of HIV-1 do not undergo nuclear import in G_0-arrested cells (4).

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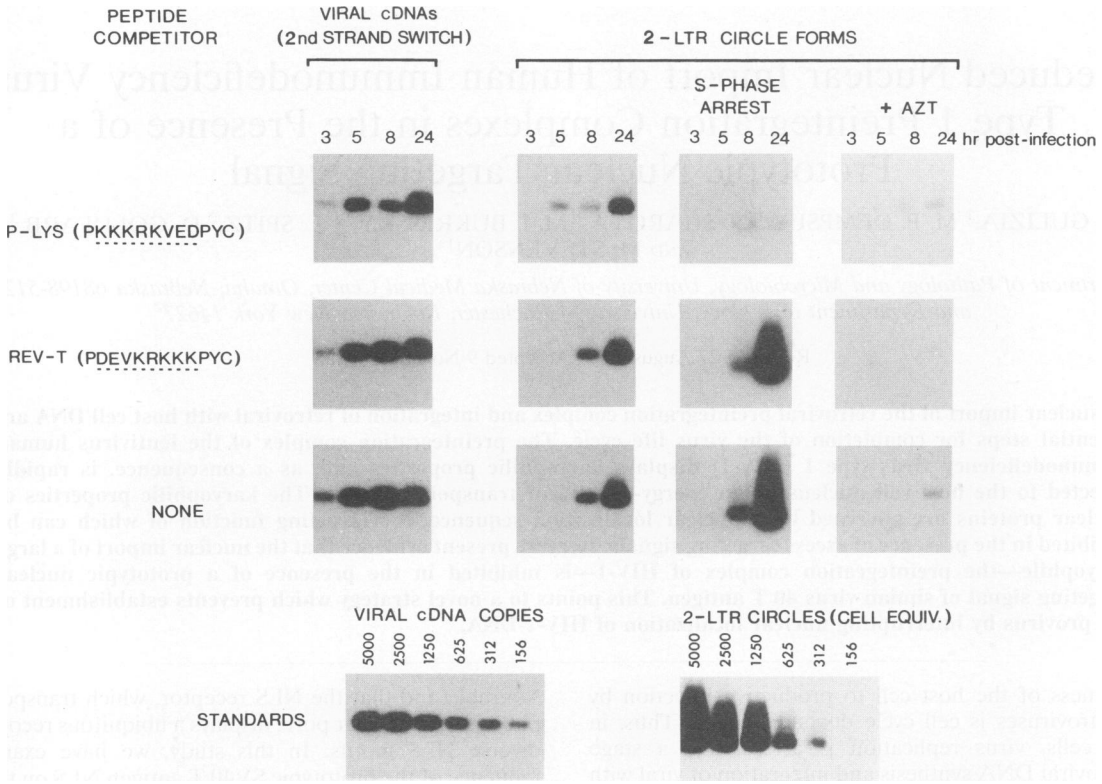

FIG. 1. HIV-1 DNA synthesis and circularization in the presence of prototypic SV40 T antigen NLS motifs. $CD4^+$ MT4 cells were growth arrested at the G_1-S interphase by treatment (18 h) with aphidicolin (4). Proliferating and growth-arrested cells were incubated for 1 h in the presence of the indicated HPLC-purified peptides (100 μM) and infected with HIV-1 isolate MF (23). At the indicated times postinfection, 0.5 $\times$ 10^6 cells were removed for analysis of viral DNA synthesis and circularization by PCR (3, 4). Cultures receiving zidovudine (AZT) were incubated in the presence of drug for 18 h prior to HIV-1 infection. Viral cDNA standards were generated from doubling dilutions of 8E5 cells (9), which contain one defective (*pol*) viral genome per cell. Viral 2 LTR circle standards were generated from doubling dilutions of HIV-1-infected MT4 cells.

Conditions for optimal aphidicolin arrest of $CD4^+$ MT4 T cells are as described previously (4). Incubation of MT4 cells in aphidicolin (5 μg/ml) for 18 h reduced DNA synthesis (measured by [^{3}H]thymidine incorporation into DNA) by approximately 90% (aphidicolin-treated MT4, 3.6 $\times$ 10^4 counts; untreated MT4, 2.85 $\times$ 10^5 counts). Cells arrested in this manner were infected with a high titer of HIV-1 (400 tissue culture infectious dose units/10^6 cells) in the presence or absence of highly purified (high-pressure liquid chromatography [HPLC]) peptide competitor (100 μM), and viral DNA synthesis and circularization were monitored over the first 24 h postinfection. In the presence of some peptide preparations which had not been purified by HPLC, viral DNA synthesis following acute infection was dramatically reduced, suggesting that these preparations interrupted a step prior to entry of the viral core (virus binding, fusion, or uncoating). This effect did not appear to sequence specific, and the basis for this inhibition by certain peptide preparations is currently under investigation.

Circularization of viral DNA is inhibited by SV40 T antigen NLS peptides. The kinetics of viral DNA synthesis and circularization in growth-arrested $CD4^+$ cells cultured in the presence of peptides containing the NLS of SV40 T antigen (P-LYS) and NLS peptides containing an inverted T antigen targeting signal (REV-T) is shown in Fig. 1. Prior to infection, the virus inoculum was treated with DNase to remove residual proviral DNA (29). The synthesis of late reverse transcription products in infected cultures maintained with or without peptides was equivalent. The amount of target viral DNA and number of PCR amplification cycles were adjusted so as to ensure that the PCR was in the linear stage of amplification and that the amount of amplification product generated was directly proportional to target viral cDNA copy number. Next, the kinetics of viral DNA circularization in G_1-S-arrested and nonarrested cultures maintained with and without peptide was compared. In both arrested and nonarrested cultures, circularization of viral DNA in the presence of REV-T NLS peptide was identical to that in cultures lacking peptide (Fig. 1). In the presence of P-LYS NLS peptides, synthesis of viral DNA in both dividing and growth-arrested cultures was equivalent to that observed in cultures containing REV-T peptide or lacking peptide. In contrast, however, circularization of viral DNA was absent in S-phase-arrested cells cultured in the presence of the P-LYS peptide (Fig. 1). Thus, the inhibitory effect of the P-LYS peptide on circularization of viral DNA, which was apparent only in nondividing cells, is consistent with the notion that this peptide interferes with the active nuclear import of HIV-1 DNA. This pattern of inhibition was observed in three independent experiments with two different peptide preparations. P-LYS peptide concentrations of 20 μM and below did not have any significant effect on either circularization of viral cDNA or on nuclear localization of viral preintegration complexes. No circle forms of viral DNA were detectable in cultures containing zidovudine (1 μm), demonstrating that all

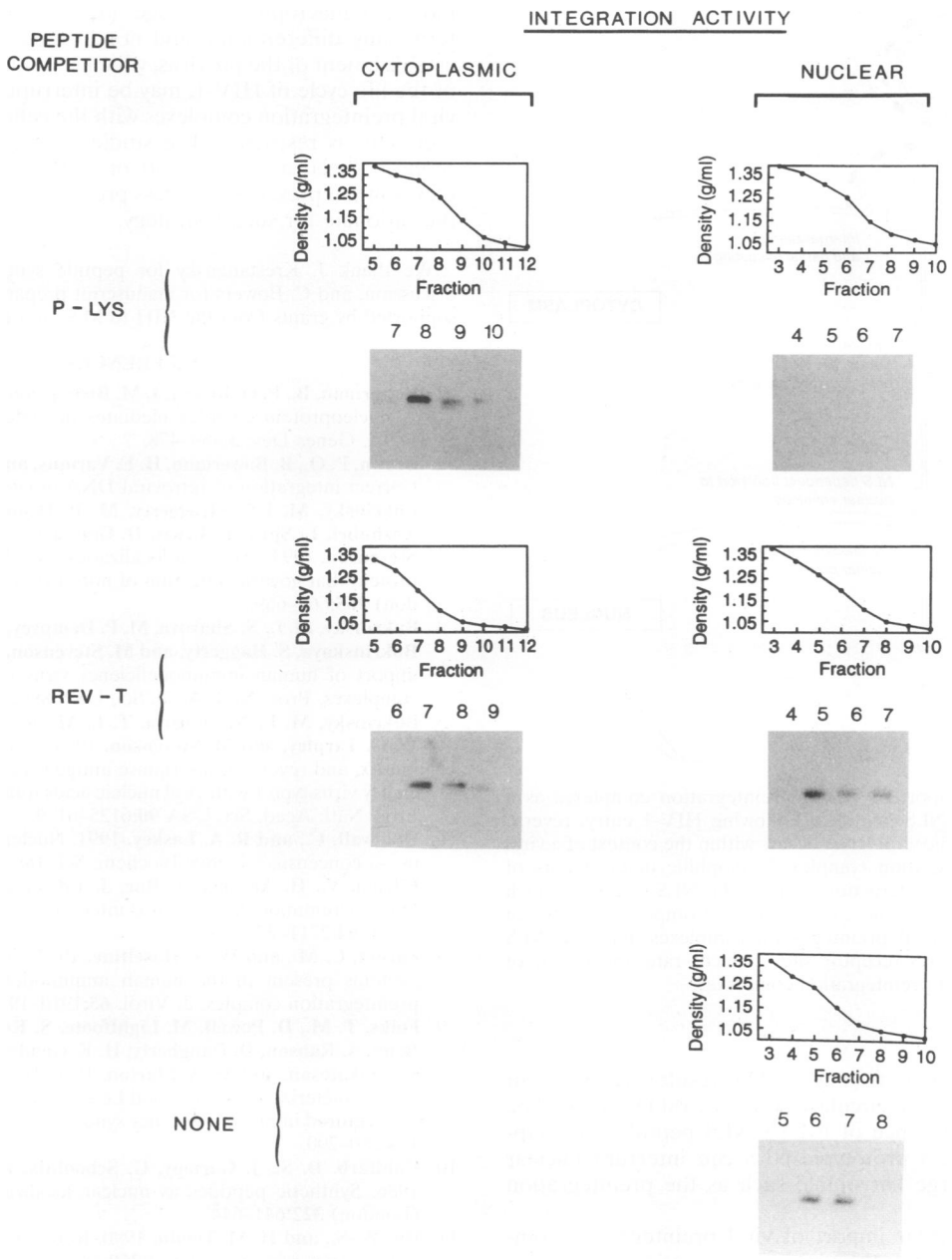

FIG. 2. Subcellular distribution of HIV-1 preintegration complexes in the presence of T antigen NLS peptides. MT4 cells were S phase arrested by aphidicolin and then infected with HIV-1. MT4 cells were harvested 15 to 18 h postinfection and lysed in hypotonic buffer (24). DNA from nuclear and cytoplasmic extracts were isolated as detailed elsewhere (18) and fractionated on nonionic density gradients, and integration activity in each gradient fraction (0.5 ml) was analyzed in vitro by using the modified protocol of Ellison et al. (7) as outlined previously (4).

amplification products were the result of de novo viral DNA synthesis rather than preexisting viral DNA present in the virus inoculum (29).

T antigen NLS peptides restrict nuclear import of HIV-1 preintegration complexes. The subcellular distribution of viral preintegration complexes (24 h postinfection) in growth-arrested $CD4^+$ cells, maintained with and without NLS peptides, is shown in Fig. 2. Nuclear and cytoplasmic cell extracts were prepared after hypotonic cell lysis and were subjected to Nycodenz gradient centrifugation as detailed elsewhere (4, 24). Gradient fractions with a density of ~1.20 to 1.25 g/ml, which contained viral cDNA (as evidenced by PCR and *pol* primers) and which would be predicted to contain retroviral preintegration complexes (1, 2, 4, 5, 8), were further identified by the presence of in vitro integration activity in each fraction (7). Integration activity in both nuclear and cytoplasmic fractions from HIV-1-infected cultures maintained in the presence of REV-T peptide was identified. In addition, this integration activity was observed in fractions of the expected density in both cytoplasmic and nuclear extracts (Fig. 2). In contrast, integration activity was detected in cytoplasmic, but not nuclear, extracts of HIV-1-infected cultures maintained in the

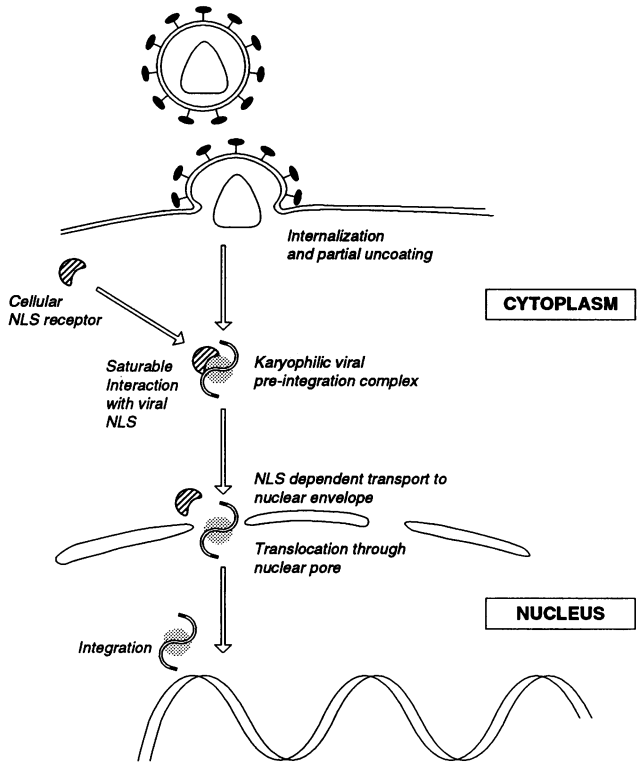

FIG. 3. Nuclear import of HIV-1 preintegration complexes as a target for prototypic NLS peptides. Following HIV-1 entry, reverse transcription of genomic viral RNA occurs within the context of a viral nucleoprotein preintegration complex. Karyophilic determinants of the complex mediate its interaction with cellular NLS receptors which direct viral complexes to the nuclear pore. Competition between karyophilic motifs of viral preintegration complexes and free NLS motifs for available NLS receptor sites reduces rate and extent of nuclear import of viral preintegration complexes.

presence of P-LYS peptide (Fig. 2). This result was consistent with the observation that circularization of viral DNA could be prevented in the presence of P-LYS NLS peptides and supports the view that a prototypic NLS can interrupt nuclear localization of a large karyophile such as the preintegration complex of HIV-1.

In this study, nuclear import of viral preintegration complexes was interrupted in the presence of peptides containing the prototype NLS motif of SV40 T antigen but not peptides containing a reverse T antigen signal. This is consistent with the function of such signals which, when conjugated to heterologous proteins, will direct their nuclear import only if presented in forward orientation (10). Transport of karyophiles to the nuclear pore is energy independent but requires the presence of a functional targeting sequence (20). Recognition of the NLS and transport of the karyophile to the nuclear pore are mediated by a cellular receptor (as illustrated in Fig. 3), and presumably the presence of excess NLS motifs competes with the karyophile for available NLS receptor sites, thus reducing rate and extent of nuclear import.

The karyophilic property of the preintegration complex of HIV-1 (4) accounts in part for the ability of this lentivirus to establish a provirus and to replicate within nondividing host cells (3, 4, 14, 27). Within non-T-cell compartments of the infected host, HIV-1 replication occurs mainly within cells of monocyte/macrophage lineage (12, 19, 22, 28) which are terminally differentiated and nondividing. Within such cells, establishment of the provirus, which is essential for completion of the life cycle of HIV-1, may be interrupted if interaction of viral preintegration complexes with the cellular nuclear import apparatus is restricted. The studies in this report which describe reduced nuclear import of HIV-1 preintegration complexes in the presence of excess prototypic NLS motifs provide the rationale for such a strategy.

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