The EMBO Journal Vol.17 No.4 pp.909–917, 1998

Viral protein R regulates nuclear import of the HIV-1 pre-integration complex

Serguei Popov[1], Michael Rexach[2], Gabriele Zybarth[1], Norbert Reiling[1], May-Ann Lee[1,5], Lee Ratner[3], Cynthia M.Lane[4], Mary Shannon Moore[4], Günter Blobel[2] and Michael Bukrinsky[1,6]

[1]The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, [2]Laboratory of Cell Biology, The Rockefeller University, Howard Hughes Medical Institute, New York, NY 10021, [3]Division of Molecular Oncology, Washington University Medical Center, St. Louis, MO 63110, [4]Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA and [5]Defense Medical Research Institute, Singapore 119260

[6]Corresponding author
e-mail: mbukrinsky@picower.edu

Replication of human immunodeficiency virus type 1 (HIV-1) in non-dividing cells critically depends on import of the viral pre-integration complex into the nucleus. Genetic evidence suggests that viral protein R (Vpr) and matrix antigen (MA) are directly involved in the import process. An *in vitro* assay that reconstitutes nuclear import of HIV-1 pre-integration complexes in digitonin-permeabilized cells was used to demonstrate that Vpr is the key regulator of the viral nuclear import process. Mutant HIV-1 pre-integration complexes that lack Vpr failed to be imported *in vitro*, whereas mutants that lack a functional MA nuclear localization sequence (NLS) were only partially defective. Strikingly, the import defect of the Vpr$^-$ mutant was rescued when recombinant Vpr was re-added. In addition, import of Vpr$^-$ virus was rescued by adding the cytosol of HeLa cells, where HIV-1 replication had been shown to be Vpr-independent. In a solution binding assay, Vpr associated with karyopherin α, a cellular receptor for NLSs. This association increased the affinity of karyopherin α for basic-type NLSs, including that of MA, thus explaining the positive effect of Vpr on nuclear import of the HIV-1 pre-integration complex and BSA–NLS conjugates. These results identify the biochemical mechanism of Vpr function in transport of the viral pre-integration complex to, and across, the nuclear membrane.

Keywords: HIV-1/karyopherin α/matrix protein/nuclear import/Vpr

Introduction

Among the different groups of retroviruses, lentiviruses are unique in their ability to infect and replicate in non-dividing cells (Varmus and Swanstrom, 1985). This property of lentiviruses, and of human immunodeficiency virus type 1 (HIV-1) in particular, depends on the active transport of their genome into the nucleus of an infected cell, without the requirement for nuclear envelope breakdown during cell division (Bukrinsky *et al.*, 1992; Lewis *et al.*, 1992; Schmidtmayerova *et al.*, 1997). Three HIV-1 proteins: matrix antigen (MA), integrase (IN) and viral protein R (Vpr), have been implicated in nuclear import of the HIV-1 pre-integration complex (PIC) (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994; Gallay *et al.*, 1997). It has been hypothesized that the HIV-1 PIC is targeted for nuclear import by interacting with the cellular machinery that executes nuclear import of the cell's own karyophiles, although the role of MA in this process was questioned in some publications (Freed *et al.*, 1995; Fouchier *et al.*, 1997).

Nuclear import of proteins that contain a basic-type nuclear localization sequence (NLS), similar to that of the SV-40 large T antigen, is mediated by karyopherin α/β heterodimers (also termed NLS receptor/p97, importin α/β, PTAC or Kap60/95) (Gorlich *et al.*, 1995). Karyopherin α binds the NLS (Adam and Gerace, 1991; Imamoto *et al.*, 1995; Moroianu *et al.*, 1995a,b; Rexach and Blobel, 1995; Weis *et al.*, 1995), whereas karyopherin β enhances the affinity of karyopherin α for the NLS (Rexach and Blobel, 1995; Moroianu *et al.*, 1996) and mediates docking of karyopherin–NLS protein complexes to nucleoporins (a collective term for nuclear pore complex proteins) containing xxFG peptide repeats (Iovine *et al.*, 1995; Kraemer *et al.*, 1995; Moroianu *et al.*, 1995b; Radu *et al.*, 1995; Rexach and Blobel, 1995; Hu *et al.*, 1996). The GTPase Ran–TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993) is probably the major regulator of the directionality of nuclear transport (Gorlich *et al.*, 1996a). The direct binding of Ran–GTP to karyopherin β terminates the translocation by disassembling the import complex (Rexach and Blobel, 1995; Chi *et al.*, 1996; Gorlich *et al.*, 1996b).

Evidence suggests that HIV-1 uses karyopherin α/β to translocate its genome into the nucleus. Gallay *et al.* (1996) showed that karyopherin α (Rch1) is bound to MA in the HIV-1 PIC. In addition, a drug that inhibits nuclear import of HIV-1 PICs prevents the interaction of the PIC with karyopherin α (Popov *et al.*, 1996). The functional role of Vpr in nuclear translocation of the HIV-1 PIC remains poorly defined. Vpr localizes to the nucleus in HIV-1-infected cells or when expressed from a vector (Lu *et al.*, 1993; Di Marzio *et al.*, 1995; Yao *et al.*, 1995). Although the C-terminus of Vpr contains several arginine residues that resemble a basic NLS (Lu *et al.*, 1993), this sequence does not function as an NLS; instead it seems that the nuclear targeting function of Vpr is located in the N-terminal region that is required for protein–protein interactions (Di Marzio *et al.*, 1995; Yao *et al.*, 1995; Mahalingam *et al.*, 1997).

Studies of the mechanisms of HIV-1 nuclear import *in vivo* are complicated by the fact that this process occurs

in conjunction with a series of other events, including uncoating of the virus and reverse transcription, which may be regulated by the same viral proteins that control nuclear import. To avoid interference from other events while staying close to the *in vivo* situation, we developed an *in vitro* system which allows investigation of HIV-1 nuclear import. This model uses digitonin-permeabilized cells and HIV-1 PICs, thus by-passing replication steps preceding PIC formation. Using this system we demonstrate the role of Vpr as the key regulator of HIV-1 nuclear import. We show that Vpr associates with karyopherin α, and that this association results in an increased binding affinity of the latter for basic-type NLSs, including that of MA. The high affinity binding appears to be critical for karyopherin α-mediated transport of large karyophiles with relatively weak NLSs, such as the HIV-1 PIC. Our results identify a novel mechanism by which Vpr regulates HIV-1 nuclear import, and suggest that proteins with a similar activity may exist in normal cells and function during nuclear import.

Results

Vpr and MA control nuclear import of the HIV-1 PIC in digitonin-permeabilized HeLa cells

To investigate nuclear import of HIV-1 PICs, we modified a recently developed assay for the *in vitro* nuclear import of the Hepadnavirus genome (Kann *et al.*, 1997). We used cytoplasmic extracts of H9 cells that were infected with wild-type HIV-1, isogenic variants that carry inactivating mutations in the MA NLS, or Vpr, as a source of HIV-1 PICs. These cell extracts contain all karyopherins and soluble factors required for nuclear import. We used H9 cells because they support replication of the mutant viruses used in this study, thus ensuring that functional PICs were present in the cytoplasmic extracts used. The efficiency of the HIV-1 import reaction was monitored by the appearance of 2-LTR HIV-1 DNA circles, which are formed exclusively within the nuclear compartment (Varmus and Swanstrom, 1985; Brown *et al.*, 1987). All the mutant PICs used are capable of forming 2-LTR circles, as seen in infected T cell lines where these viruses replicate effectively (data not shown; Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994). The total amount of viral PICs was quantified after immunoprecipitation with antiserum against MA by PCR, using primers specific for the HIV-1 *pol* gene. This PCR-based quantitation system is linear in relation to HIV-1 DNA (Figure 1A, bottom). All cytoplasmic extracts used contained similar amounts of HIV-1 PICs (Figure 1A, top). A similar result was obtained when antisera against integrase or reverse transcriptase were used to precipitate the PIC (data not shown), indicating that the lack of Vpr, or mutations in MA, did not grossly change the composition of the PIC. No 2-LTR forms of the HIV-1 DNA were detected in these extracts (Figure 1A, second panel). To assess damage to nuclei during *in vitro* reactions, permeabilized cells were incubated with FITC–WGA, or with FITC–Concanavalin A to stain ruptured nuclear envelopes (Dean and Kasamatsu, 1994). Most nuclei (~95%) did not stain with FITC–ConA and all nuclei stained brightly with FITC-WGA (data not shown).

Cytoplasmic extracts from T cells containing wild-type

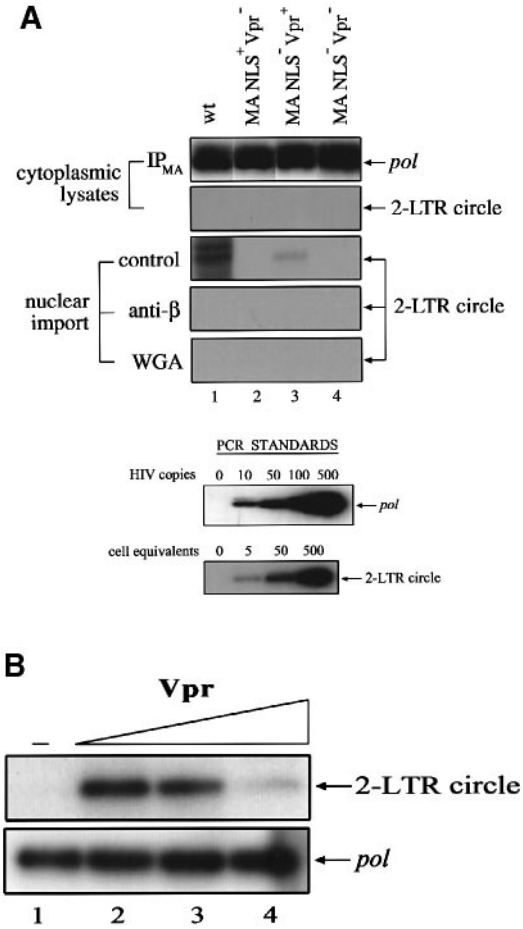


Fig. 1. The effect of Vpr on nuclear import of the HIV-1 PIC. (**A**) Mutations in Vpr and MA reduce the nuclear import efficiency of HIV-1 PICs. Cytoplasmic extracts of H9 cells containing equal amounts of wild-type HIV-1_{NLHX} (wt, lane 1) or variants with inactivating mutations in Vpr (MA NLS^+ Vpr^- lane 2), MA NLS (MA NLS^-Vpr^+, lane 3), or Vpr and MA (MA NLS^-Vpr^-, lane 4), were divided into two sets of aliquots. The PICs were immunoprecipitated from one set using sheep anti-MA polyclonal serum and protein G–Sepharose; immunoprecipitated viral DNA was analyzed by PCR using primers specific for the HIV-1 *pol* gene (to detect total HIV DNA) (top panel). Another aliquot from the first set was directly assayed for 2-LTR circle forms of HIV-1 DNA (second panel). The second set of aliquots was incubated for 1 h at 37°C with digitonin-permeabilized HeLa cells in the presence of anti-karyopherin β antibodies (anti-β), WGA or control antibodies (anti-β-amyloid antibodies). DNA was then extracted from the permeabilized cells and was analyzed by PCR with primers specific for HIV-1 2-LTR circles. The linearity of quantitation of the PCR-based assay was confirmed by amplification of HIV-1 DNA standards (bottom panels). Dilutions of 8E5 cells (Folks *et al.*, 1986) that contain one copy of HIV-1 DNA per cell were used to generate *pol* standards, whereas standards for circle viral DNA forms were generated by PCR on log dilutions of HIV-1-infected H9 cells. (**B**) Nuclear import of Vpr-defective HIV-1 can be rescued by adding recombinant Vpr. Cytoplasmic lysate of H9 cells infected with the MA NLS^+ Vpr^- virus was incubated with digitonin-permeabilized HeLa cells in the absence of Vpr (lane 1), or in the presence of recombinant Vpr at a concentration of 100 ng/ml (lane 2), 500 ng/ml (lane 3) or 5000 ng/ml (lane 4). After 1 h at 37°C, DNA was analyzed by PCR using primers specific for HIV-1 2-LTR circles or the *pol* gene.

or mutant HIV-1 PICs were incubated with digitonin-permeabilized HeLa cells for 1 h at 37°C in the presence of an energy regeneration system. 2-LTR circles were formed efficiently in cells incubated with the wild-type PICs (Figure 1A, control panel, lane 1). Formation of

2-LTR circles was blocked by specific inhibitors of nuclear import, including a monoclonal antibody to karyopherin β (Figure 1A, anti-β panel, lane 1), wheat-germ agglutinin (Figure 1A, WGA panel, lane 1), and ATP depletion (data not shown). This indicates that formation of the 2-LTR circle form of HIV-1 DNA *in vitro* depends on the transport of HIV-1 DNA across the nuclear pore complex. Although wild-type PICs were imported efficiently (Figure 1A, control panel, lane 1), an inactivating mutation in the MA NLS diminished the efficiency of import (Figure 1A, compare lanes 1 and 3). A densitometry scan of the film revealed a 65% reduction in efficiency. In contrast, mutant PICs that lack Vpr were completely defective for import even in the presence of a functional MA NLS (Figure 1A, lane 2). As expected, a double mutant was also defective for import (Figure 1A, lane 4).

Added-back Vpr can rescue nuclear import of the vpr-defective virus

Since mutation in the *vpr* gene had such a dramatic effect on nuclear import of the HIV-1 PIC, we investigated whether addition of recombinant Vpr protein would rescue import of a Vpr-defective virus. Cytoplasmic lysate from T cells containing PICs of MA NLS^+Vpr^- virus (the same as used in Figure 1A) was incubated with digitonin-permeabilized HeLa cells in the presence of various concentrations of Vpr. After a 1 h, 37°C incubation, DNA was analyzed by PCR with primers specific either for the HIV-1 *pol* gene (reflecting the total amount of viral DNA in the samples), or for 2-LTR circles of viral DNA (reflecting efficiency of nuclear import). At low concentrations (100 ng/ml and 500 ng/ml, Figure 1B, lanes 2 and 3) Vpr rescued nuclear import of this mutant, as revealed by the appearance of 2-LTR circles. However, this effect disappeared at a higher concentration of Vpr (5 μg/ml, Figure 1B, lane 4). The biphasic nature of the Vpr activity argues against a non-specific effect due to contaminations from the baculovirus expression system. Furthermore, addition of a lysate of baculovirus-infected Sf9 cells to our assay did not affect nuclear import (data not shown).

This result underlines an important difference between the effects of Vpr and the MA NLS on HIV-1 nuclear import. Vpr does not have to be a part of the PIC to exert its enhancing effect, while this is a critical requirement for MA. Indeed, addition of recombinant MA or NLS peptides did not improve nuclear import of the MA NLS-defective virus (data not shown). Therefore, while MA via its NLS seems to be the driving force of the HIV-1 nuclear import, Vpr appears to be the key regulator of this process.

Cytoplasmic lysate from HeLa cells can rescue import of the vpr-defective virus

The essential role of Vpr in our *in vitro* nuclear import system seems to contradict the results of Gallay *et al.* (1997) who reported Vpr-independent replication and nuclear import of HIV-1 in HeLa cells. It is important to note that in our system digitonin-permeabilized HeLa cells provided only nuclei and cytoskeleton, while all soluble components of transport came from T cells. We therefore tested cytoplasmic extracts from various cells for the ability to rescue nuclear import of the *vpr*-defective HIV-1. Addition of cytoplasmic lysate from HeLa cells (Figure 2, lane 4) restored nuclear import of Vpr^- HIV-1, similar to the effect of recombinant Vpr (Figure 2, lane 5). Import of Vpr^- virus was not rescued by cytoplasmic lysates from resting (Figure 2, lane 1) or activated (Figure 2, lane 2) T cells, nor from macrophages (Figure 2, lane 3) where HIV-1 replication is Vpr-dependent (Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994).

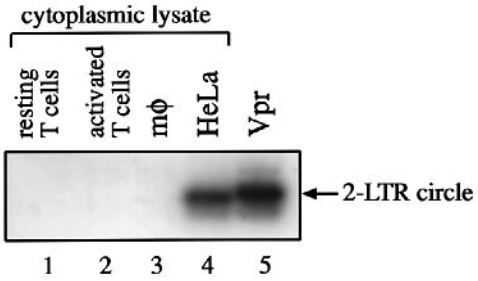


Fig. 2. Nuclear import of Vpr-defective HIV-1 can be rescued by cytosol of HeLa cells. Cytoplasmic lysate of H9 cells infected with the MA NLS^+ Vpr^- virus (same as in Figure 1B) was diluted 1:1 with the cytosol (protein concentration 0.5 mg/ml) of resting primary T cells (lane 1), PHA+IL-2 activated T cells (lane 2), macrophages (lane 3) or HeLa cells (lane 4) and used in the import assay as in Figure 1. In lane 5, recombinant Vpr (0.5 μg/ml) was added to the import reaction. DNA was analyzed as in Figure 1 using primers specific for 2-LTR circles.

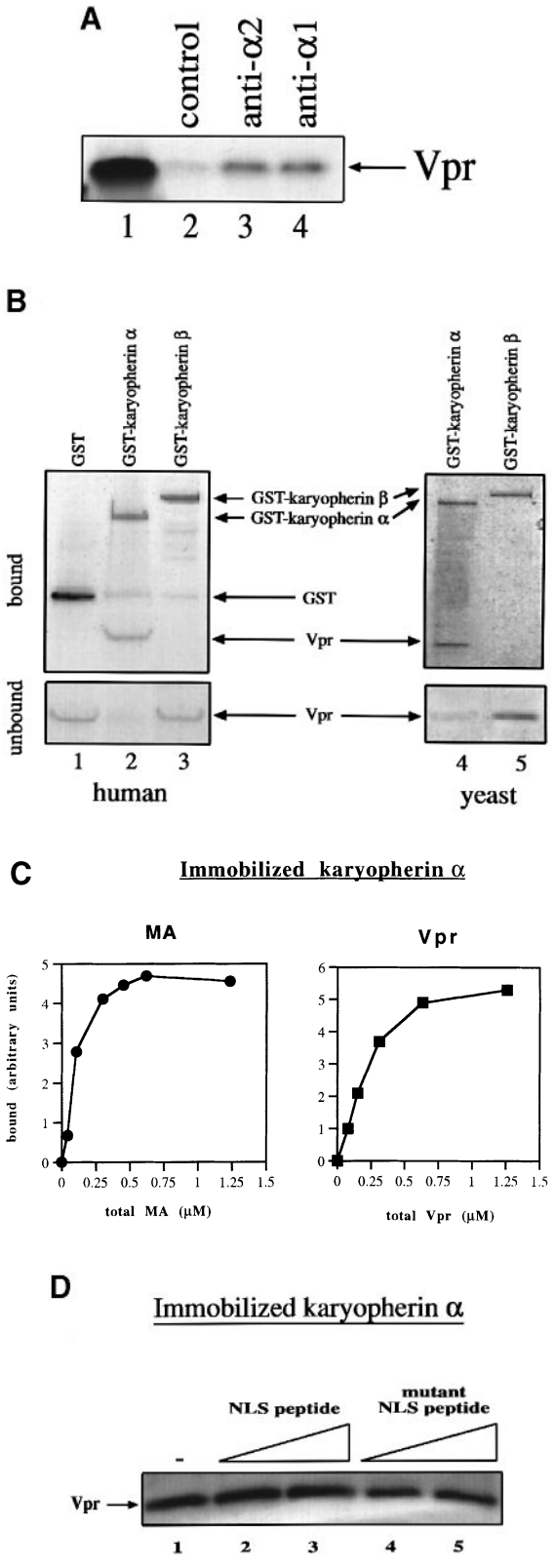
Vpr binds to karyopherin α

Evidence suggests that HIV-1 uses karyopherin α/β heterodimers to translocate its genome into the nucleus (Gallay *et al.*, 1996; Popov *et al.*, 1996). We therefore tested whether Vpr exerts its effect via binding to karyopherin α/β. Radiolabeled Vpr was expressed in a reticulocyte lysate transcription–translation system, and aliquots were subjected to immunoprecipitation using control IgGs or affinity-purified antibodies against human karyopherin α2 (Rch1) and karyopherin α1 (hSrp1) (Nadler *et al.*, 1997). As shown in Figure 3A, Vpr was precipitated from the lysate by the anti-karyopherin α antibodies, but not by control IgGs. This result supports the notion that Vpr binds to karyopherin α/β.

To examine the possible interaction of Vpr with karyopherin α/β in greater detail, we used a solution binding assay with purified Vpr, karyopherin α and karyopherin β. Immobilized GST-karyopherin α2 (Rch1) or GST-karyopherin β (p97) were incubated with Vpr. As shown in Figure 3B (left panels), Vpr bound to immobilized karyopherin α (Figure 3B, lane 2), but not to karyopherin β (Figure 3B, lane 3) or GST (Figure 3B, lane 1). Vpr binds to a domain in karyopherin α that has been conserved through evolution, as similar results were obtained using recombinant yeast karyopherins (Figure 3B, right panel). We calculated the binding affinity of Vpr and MA for karyopherin α (Rch1). Binding of both HIV-1 proteins to yeast karyopherin α was saturable, with half-maximal binding achieved at ~0.19 μM for Vpr (Figure 3C, right panel), and ~0.1 μM for MA (Figure 3C, left panel).

We tested whether the interaction of Vpr with karyopherin α is mediated by a basic-type NLS. Vpr was pre-incubated with karyopherin α to allow formation of the complex, followed by incubation with the competing peptide corresponding to the SV-40 large T antigen NLS.

Binding of Vpr to karyopherin α was resistant to competition with the NLS peptide (Figure 3D); even a 1000-fold excess of the NLS peptide did not displace Vpr from the complex with karyopherin α (Figure 3D, lane 3). This result suggests that Vpr binding to karyopherin α does not involve basic-type NLS.



Vpr increases the affinity of NLS-karyopherin α interaction

NLS-independent binding of Vpr to karyopherin α suggested that Vpr can bind to karyopherin α simultaneously to MA, binding of which was shown to be dependent on basic-type NLS (Gallay *et al.*, 1996; Popov *et al.*, 1996). This was indeed the case (Figure 4, bottom panel); most strikingly, the interaction of MA with karyopherin α became resistant to competition by the NLS peptide when Vpr was present (Figure 4, bottom panel). A decrease in the amount of bound MA was observed only at a 2000-fold excess of the peptide (Figure 4, lane 5). This decrease in MA binding at high peptide concentration suggests that a higher affinity interaction between MA and karyopherin α in the presence of Vpr is still NLS-dependent. Consistent with this conclusion, MA with a mutagenized NLS did not bind to karyopherin α, irrespective of the presence or absence of Vpr (data not shown). In addition, Vpr and MA did not bind to each other, nor compete with each other for the binding site on karyopherin α (data not shown). These results suggest that Vpr positively regulates the affinity of karyopherin α for the NLS of MA.

Vpr stimulates nuclear import of an artificial weak karyophile

One of the anticipated consequences of Vpr function would be to affect nuclear import of other karyophiles. To test this prediction, we conjugated rhodamine-labeled BSA with an SV-40 T antigen NLS-peptide, a prototype for basic-type import signals. Conjugation was performed at different peptide:BSA ratios, resulting in import substrates with different karyophilic activities (Dworetzky *et al.*, 1988). Nuclear import of these karyophiles was tested in digitonin-permeabilized cells in the presence and absence of recombinant Vpr. At concentrations of 50 and 100 ng/ml Vpr significantly enhanced nuclear import of a weak karyophile (Figure 5A and B) without affecting import of a strong karyophile (Figure 5A). At higher concentrations, Vpr significantly inhibited nuclear import

Fig. 3. Analysis of Vpr–karyopherin interactions. (**A**) Vpr associates with mammalian karyopherin α in cell extracts. Vpr was transcribed and translated *in vitro* in the presence of ^{35}S-methionine and ^{3}H-leucine (lane 1). A translation reaction was divided into three aliquots which were subjected to immunoprecipitation with affinity purified anti-karyopherin α1 (hSrp1) (lane 4), anti-karyopherin α2 (Rch1) (lane 3) or preimmune rabbit IgG (lane 2). Precipitates were analyzed on SDS–PAGE, followed by fluorography of the gel. (**B**) Vpr binds directly to karyopherin α, but not to karyopherin β. Vpr (0.6 μg) was mixed with 1 μg of immobilized GST (lane 1), GST-karyopherin α (human Rch1, lane 2; or yeast Kap60, lane 4) or GST-karyopherin β (human p97, lane 3; or yeast Kap95, lane 5). Reactions were incubated for 20 min at room temperature. After washing the beads, proteins in the bound and unbound fractions were resolved by SDS–PAGE and stained with Coomassie Blue. (**C**) Kinetics of interaction between MA, Vpr and karyopherin α. Immobilized GST-karyopherin α (Rch1) was mixed with various amounts of Vpr (left panel) or MA (right panel) in a 60 μl reaction. After 20 min at room temperature the beads were washed and proteins in the bound fraction were resolved by SDS–PAGE and stained with Coomassie Blue. The amount of bound protein was quantified by gel densitometry. (**D**) Binding of Vpr to karyopherin α cannot be competed with an NLS peptide. Recombinant Vpr (0.2 μg or 14 pmol) was incubated with immobilized GST-karyopherin α (Rch1) (0.6 μg or 6.5 pmol) for 20 min at room temperature, followed by a 20 min incubation with no peptide (lane 1), NLS peptide (4.8 and 14 nmol, lanes 2 and 3, respectively) or mutant NLS peptide (4.8 and 14 nmol, lanes 4 and 5, respectively). Proteins in the bound fraction were resolved by SDS–PAGE and stained with silver.

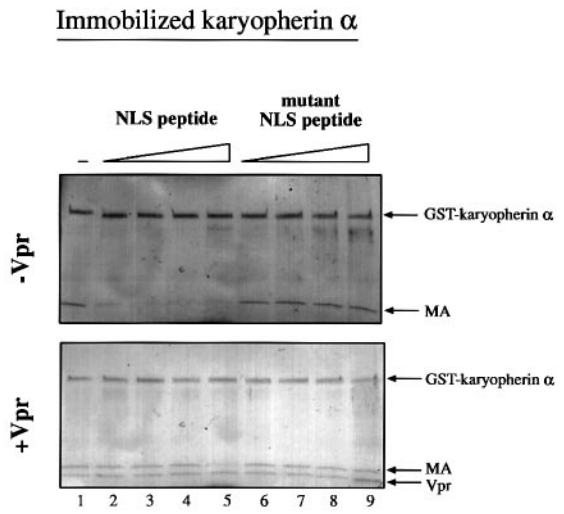


Fig. 4. Competition analysis of MA and Vpr interaction with mammalian karyopherin α. Recombinant MA (12 pmol) was incubated with immobilized GST–Rch1 in the absence (upper panel) or presence (bottom panel) of Vpr (57 pmol) for 20 min at room temperature, followed by a 20 min incubation with no addition (lane 1), 1.2 nmol (lanes 2, 6), 4.8 nmol (lanes 3, 7), 12 nmol (lanes 4, 8) and 24 nmol (lanes 5, 9), of NLS peptide (lanes 2–5) or mutant NLS peptide (lanes 6–9). Proteins in the bound fraction were resolved by SDS–PAGE and stained with Coomassie Blue.

of all the karyophiles (Figure 5A and B). This observation is consistent with the inhibitory effect of high concentrations of Vpr on reconstituted import of HIV-1 PICs (Figure 1B, lane 4).

Discussion

Several lines of evidence presented in this paper indicate that Vpr plays a critical role in the regulation of HIV-1 nuclear import. First, inactivating mutations in the *vpr* gene resulted in a greatly diminished nuclear translocation of the HIV-1 PIC in our *in vitro* system. Secondly, added-back recombinant Vpr rescued nuclear import of a Vpr-defective HIV-1 and stimulated import of an artificial weak karyophile. Thirdly, Vpr associated with karyopherin α, and this binding increased the affinity of interaction between karyopherin α and NLS-containing proteins. Our data suggest that Vpr regulates the nuclear import of HIV-1 PIC by binding to karyopherin α and increasing its affinity for viral NLSs, including the NLS of MA. This binding interaction may allow the PIC to compete efficiently for karyopherin α/β heterodimers in the cytosol and thus may facilitate docking and movement of the PIC across the NPC. Without Vpr, the nuclear import of such complexes is greatly impaired (Figure 1). In contrast, mutation of the MA NLS had only a modest effect on nuclear import of the HIV-1 PIC (Figure 1), probably because there are other NLSs in the PIC (discussed below).

The PCR-based assay used for monitoring nuclear import of the HIV-1 PIC in this study relies on detection of 2-LTR circle forms of the HIV-1 DNA. Although these forms of viral DNA do not appear to be precursors of the integrated provirus (Brown *et al.*, 1989; Bukrinsky *et al.*, 1993), they are convenient markers for nuclear import since they are produced from the same cytoplasmic PIC as the integration precursor (Bukrinsky *et al.*, 1993), following completion of reverse transcription and translocation of these PICs into the nucleoplasm (Brown, 1990; Bukrinsky *et al.*, 1992). Beside high sensitivity and specificity, the ability of this method to discriminate between perinuclear (i.e. bound to the outside of the nuclear envelope) and intranuclear localization of the PIC makes it vastly superior to other techniques, which rely on cell fractionation and detection of PIC-associated HIV-1 proteins.

The requirement for Vpr in reconstituted nuclear import (Figure 1) correlates well with the data obtained from *in vivo* experiments which demonstrate a critical role of Vpr in HIV-1 infection of macrophages (Westervelt *et al.*, 1992b; Balotta *et al.*, 1993; Balliet *et al.*, 1994; Connor *et al.*, 1995). It is also consistent with the observation that Vpx, which is the SIV homologue of the HIV-1 Vpr, is the critical determinant of nuclear import of SIV_{mac} in macrophages (Fletcher *et al.*, 1996). Even though most published reports agree on the role of Vpr in HIV-1 infection of non-dividing cells, the magnitude of this effect clearly differs between experimental systems. The fact that substantial replication (and nuclear import) of HIV-1 with a mutation in the *vpr* gene was observed in growth-arrested HeLa cells (Emerman *et al.*, 1994; Gallay *et al.*, 1997) suggests that Vpr is not required for import under all circumstances. A discrepancy between the absolute requirement for Vpr in our *in vitro* nuclear import system and an apparently non-essential role of this protein in some *in vivo* settings may be explained by the presence of putative cellular analogues of Vpr. Our result showing that nuclear import of a Vpr^- HIV-1 can be rescued by the cytosol of HeLa cells (Figure 2) supports this hypothesis. This Vpr-like protein(s) may be induced by various treatments used in the *in vivo* experiments, such as those employed to induce growth-arrest, thus accounting for the partly Vpr-independent nuclear import observed in growth-arrested MT4 cells (Heinzinger *et al.*, 1994). Existence of such proteins is also suggested by the amazing conservation of the Vpr binding site(s) on α karyopherins from such distantly related species as yeast and humans. One of the possible candidates for this role is the heat-shock protein 70 (Hsp70, Hsc70), which can perform functions similar to those of Vpr. Hsp70 has been shown to facilitate the interaction between the NLS and karyopherin α (Shulga *et al.*, 1996; Nadler *et al.*, 1997); the ectopic expression of human Hsp70 in mouse cells complements the defective import of a mutant SV-40 large T antigen (Jeoung *et al.*, 1991), and the depletion of Hsp70 from cytosolic extracts prevents import (Shi and Thomas, 1992; Okuno *et al.*, 1993). It seems likely that cellular Vpr-like proteins may function to enhance nuclear import in some cells in response to certain stimuli.

Although there are multiple copies of MA in each HIV-1 PIC, these may not be sufficient to support efficient import of the PIC due to its very large size (Bukrinsky *et al.*, 1993b; Miller *et al.*, 1997), and because MA is a poor karyophile on its own (Fouchier *et al.*, 1997). Vpr may thus function to enhance the interaction between karyopherin α/β and various weak basic-type NLSs in the PIC. Indeed, the enhancing effect of Vpr on NLS–karyopherin α interactions is not restricted to the NLS of MA, but affects any protein with a basic-type NLS (Figure 5). Mutations in the principal NLS of MA may be

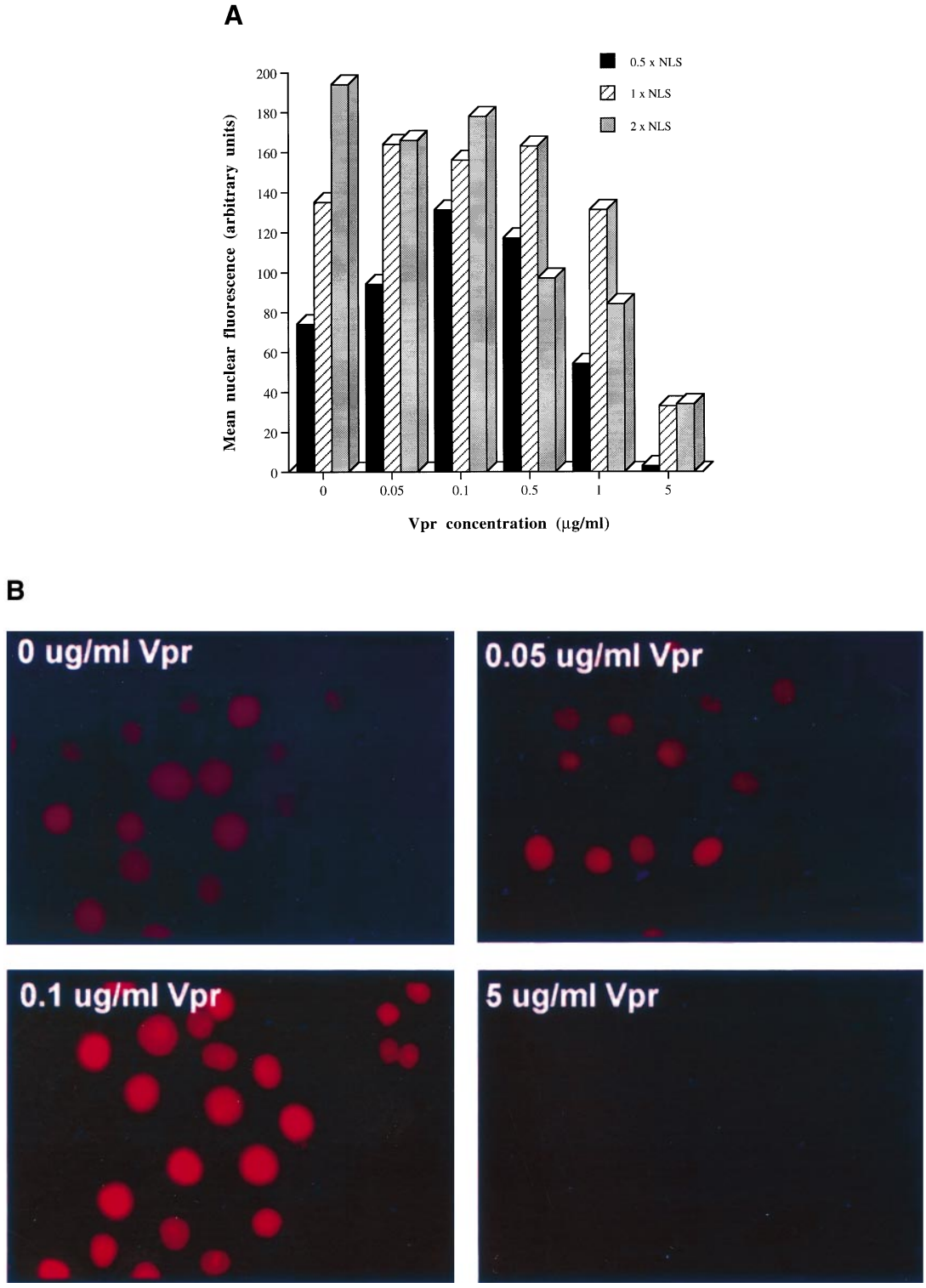


Fig. 5. Vpr affects nuclear import of an artificial karyophile. (**A**) Effect of Vpr on import of various karyophiles into the nucleus of permeabilized cells. Digitonin-permeabilized BRL cells were incubated with rhodamine-labeled NLS–BSA, recombinant human karyopherin α (20 μg/ml), karyopherin β (25 μg/ ml), Ran (15 μg/ml) and p10 (3 μg/ml), and different amounts of Vpr under standard import conditions. After 15 min at room temperature the cells were washed and fixed. Quantitation of intranuclear fluorescence was performed as described (Moore and Blobel, 1992). The NLS number indicates the molar ratio of peptide added to sulfo–SMCC-activated BSA during preparation of NLS–BSA; the exact number of NLSs conjugated to BSA could not be determined. (**B**) Fluorescence micrographs of representative nuclei after import reactions described in (A). Fluorescence micrographs show results of experiments performed with the conjugate having the lowest molar ratio of NLS peptide to BSA. Import reactions contained indicated amounts of Vpr.

compensated by other NLSs in the HIV-1 PIC. One such additional NLS is located in the C-terminus of MA (^{108}QNKSKKKA^{115}) and can bind to karyopherin α, although with a lower affinity than the principal MA NLS (^{26}GKKKYKLK^{33}) (Nadler *et al.*, 1997). Another NLS-containing protein in the HIV-1 PIC is integrase (IN),

which also binds to karyopherin α in a basic NLS-dependent manner (Gallay *et al.*, 1997). The existence of multiple NLSs regulated by Vpr may explain the controversy regarding the role of the MA NLS in HIV-1 nuclear import and replication in non-dividing cells (Bukrinsky *et al.*, 1993a; Freed *et al.*, 1995; Fouchier *et al.*, 1997).

MA and Vpr interact with karyopherin α in distinct ways. Binding of MA to karyopherin α is mediated by the NLS of MA (Figure 4), whereas binding of Vpr to α does not involve a basic-type NLS (Figure 3D). The binding site of Vpr on karyopherin α does not appear to overlap with the NLS or karyopherin β binding sites (Figure 3D and data not shown); in fact karyopherin α, Vpr and MA can assemble into a trimer (Figure 4). Our results also underlie the difference between Vpr and NLS-containing proteins in regulation of HIV-1 nuclear import. While NLS-proteins (MA and IN) serve as vehicles that drive the HIV-1 PIC into the nucleus, Vpr appears to control their function by regulating their interaction with karyopherin α. In the absence of NLS-proteins, Vpr is not expected to facilitate nuclear import. Consistent with this prediction, a novel NLS-targeting compound CNI-H0294 potently inhibited nuclear import and replication of Vpr-positive HIV-1 strains (Dubrovsky *et al.*, 1995), despite its inability to block Vpr–karyopherin α interaction (Popov *et al.*, 1996). The proposed model appears to be inconsistent with the recent observation by Gallay *et al.* (1996) that a truncated form of karyopherin α (Rch1) inhibited import of the Vpr-defective, but not the wild-type, HIV-1 PIC. A possible explanation is that Vpr has an additional, karyopherin α-independent effect on nuclear import. At the present time we cannot discount this possibility, but our preliminary results (S.Popov and M.Bukrinsky, unpublished) indicate that the Vpr-mediated stimulation of HIV-1 nuclear import requires the presence of functional NLS-proteins. We therefore conclude that Vpr is the key regulator of HIV-1 nuclear import.

As Vpr is tethered to the HIV-1 PIC early during infection, its activity probably does not affect interactions between karyopherin α and other cellular NLS-proteins. The concentration of Vpr *in vivo* during virus entry is estimated to be <5 nM, even if one makes grossly overestimating assumptions that: (i) equimolar amounts of p24 and Vpr proteins exist in an HIV-1 virion; (ii) 1% of the virions are infective and can enter the cells; (iii) cells are infected at a high multiplicity of 10 μg/ml of p24. However, at later stages of viral replication a high-level of expression of Vpr may contribute to the cytopathic effect of HIV-1 infection. Indeed, Vpr is a potent inhibitor of nuclear import at high concentrations (>42.8 nM Vpr) (Figures 1B and 5) and may block transport by 'clogging' the NPC with substrates bound too tightly to karyopherins, or by affecting recycling of karyopherin α to the cytoplasm.

In summary, our experiments suggest that Vpr regulates HIV-1 nuclear import by enhancing the interaction of karyopherin α with the NLSs of the PIC, thus making the HIV-1 PIC a strong karyophile.

Materials and methods

HIV-1 variants, infections and preparation of cytoplasmic extracts

HIV-1 variants used in this work were based on the NLHX virus strain (Westervelt *et al.*, 1992b). The mutant virus MA NLS$^-$ contains substitutions of isoleucine residues for lysines in positions 26 and 27 of MA, thus inactivating the NLS (Bukrinsky *et al.*, 1993a; von Schwedler *et al.*, 1994). In NLHXΔVpr virus the initiating ATG of the *vpr* gene was changed to GTG, and in NLHXdVpr a frameshift mutation was introduced at codon 63 of Vpr (Westervelt *et al.*, 1992a); both mutations abolished expression of Vpr. All viruses were obtained from supernatants of Jurkat cells transfected with molecular clones. Infections of H9 cells were performed at viral titers equalized according to p24 content. Cells were incubated with virus (100 ng of p24 per 10^6 cells) for 2 h at 37°C, then washed three times and incubated for an additional 4–5 h at 37°C to allow reverse transcription to proceed. Cytoplasmic extracts were prepared by lysis of cells in cold, hypotonic buffer [10 mM KCl, 10 mM Tris–HCl (pH 7.6), 0.5 mM $MgCl_2$, 1 mM DTT, 1 μg/ml of leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)] by 20–30 strokes of a Dounce homogenizer. Cell lysis was monitored by phase-contrast microscopy. After removal of nuclei, cytoplasmic extracts containing the HIV-1 PIC (Bukrinsky *et al.*, 1993b) were cleared by centrifugation at 15 000 *g* for 10 min, and aliquots were frozen at –70°C.

Expression of recombinant Vpr and MA

A baculovirus expressing His-tagged Vpr was obtained from Dr N.Landau (ADARC, Rockefeller University). One ml of an Sf9 cell lysate (10 mg/ml of protein) was incubated with 60 μl of NTA matrix (Quiagen) in binding buffer (10 mM Tris, pH 8.0, 2% NP-40, 0.5% DOC, 150 mM NaCl, 10 mM imidazole, 0.1 mM PMSF) for 30 min at 4°C. The beads were washed with binding buffer and twice with PBS, and Vpr was eluted with 100 μl of elution buffer (0.4 M imidazole, 1 mM 2-mercaptoethanol in PBS), followed by a second elution with 50 μl of elution buffer. Imidazole was removed by dialysis against PBS, supplemented with 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin. The concentration of Vpr obtained by this method was 10–50 μg/ml.

The MA-encoding sequence was amplified by PCR from pNL4-3 (Adachi *et al.*, 1986) using primers 5′-ATCGAATTCATGGGTGCGAG-AGGGTCGG-3′ (sense) and 5′-CTTGAATTCTTTTGGCTGACCTGG-GTGTTG-3′ (anti-sense) and ligated into vector pGEX-4T (Pharmacia) at the *Eco*RI site. The resulting plasmid was introduced into the *Escherichia coli* strain BLR (Novagen). The GST–MA fusion protein was purified on glutathione–agarose beads according to manufacturer's (Pharmacia) instructions. To obtain non-fused MA, GST–MA immobilized on agarose was cleaved with thrombin (4 NIH units for every 200 μg of chimera) for 10 min at room temperature. After thrombin inactivation with a 1.5 M excess of hirudin (Sigma), beads were precipitated and supernatant was dialyzed against PBS and concentrated on Centricon 10 (Amicon) microconcentrator.

Nuclear import assays

Nuclear import of HIV-1 preintegration complexes. HeLa cells grown to 70% confluency were washed with serum-free medium and permeabilized for 5 min at 37°C in serum-free mixed medium (1.972% 199 Eagle medium, 2% $NaHCO_3$, 20 mM L-glutamine, 1× non-essential amino acids, 10 mM sodium pyruvate) supplemented with 40 μg/ml of digitonin. After permeabilization, cells were washed twice with transport buffer (2 mM MgOAc, 20 mM HEPES pH 7.3, 110 mM KOAc, 5 mM NaOAc, 1 mM EGTA and 2 mM DTT), harvested using a plastic scraper, and resuspended in transport buffer at 5×10^6 cells/ml. For each assay, 34 μl of cells were incubated with cytoplasmic lysates in a 100 μl reaction containing 50 mM of creatine phosphate, 5 U creatine phosphokinase, 1 mM ATP and 0.1 mM dNTPs for 1 h at 37°C. At the end of incubation, cells were extracted and subjected to PCR analysis using primers specific for the *pol* gene or 2-LTR circle forms of the HIV-1 DNA (Bukrinsky *et al.*, 1992). To test for the intactness of nuclei in the transport reaction, we used an assay based on differential staining of native and compromised nuclei with FITC–WGA or FITC–ConA (Dean and Kasamatsu, 1994).

Nuclear import of rhodamine labeled BSA–NLS conjugates. NLS conjugates were prepared as described (Moore and Blobel, 1992), except with BSA instead of HSA. NLS-containing peptides were incubated with sulfo-SMCC (Pierce) activated BSA at 0.5-, 1- and 2-fold molar ratios relative to BSA. The import assay was performed in permeabilized buffalo rat liver (BRL) cells as described (Moore and Blobel, 1992). The import assay contained the following recombinant import factors: karyopherin α (20 μg/ml), karyopherin β (25 μg/ml), Ran (15 μg/ml) and p10 (3 μg/ml) in a buffer composed of 20 mM HEPES–KOH pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 1 mM GTP and 2 mM DTT. Recombinant import factors were isolated according to E.Schwoebel and M.S.Moore (manuscript in preparation). *Escherichia coli* expression vectors were obtained as follows: His-tagged human

karyopherin α (clone hSRP1α) from A.Lamond (EMBL, Heidelberg, Germany) (Weis *et al.*, 1995); human karyopherin β from D.Görlich (Universität Heidelberg); human Ran from M.Rush (NYU Medical Center, New York) (Ren *et al.*, 1995); and human p10 (pp15 expression clone) from U.Grundmann (Behringwerke, Marburg, Germany) (Grundmann *et al.*, 1988). For import assays, baculovirus-expressed His-tagged Vpr (see above) was dialyzed against transport buffer.

Solution binding assays

Solution binding assay with all recombinant proteins. The solution binding assay was performed as described previously (Rexach and Blobel, 1995) using bacterially expressed MA (see above), recombinant human GST-Rch1 (karyopherin α) (Nadler *et al.*, 1997), human GST-p97 (karyopherin β) (Chi *et al.*, 1995), Vpr (see above) and yeast karyopherins and nucleoporins (Rexach and Blobel, 1995).

NLS peptide competition analysis. The peptide corresponding to the NLS of the SV-40 large T antigen (TPPKKKRKVEDP) (Kalderon *et al.*, 1984) was used as a competitor for binding to karyopherin α. A peptide with a mutant NLS (TPPKTKRKVEDP) served as control. The peptides were added after a 20 min pre-incubation of a tested protein with karyopherin α (to allow formation of the complex), and incubation was then continued for an additional 20 min at room temperature.

In vitro translation of Vpr and immunoprecipitation

Radiolabeled Vpr was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega). The template for transcription was a PCR fragment amplified from vector pET-vpr-T (Zhao *et al.*, 1994) using the upstream primer 5′-ATGCGTCCGGCGTAGA-3′ and the downstream primer 5′-GCTAGTTATTGCTCAGCGG-3′. Approximately 0.6 μg of the PCR product was translated in a 50 μl reaction in the presence of ^{35}S-methionine and ^{3}H-leucine. The translation reaction was adjusted to a final concentration of 20 mM HEPES pH 6.8, 150 mM KOAc, 2 mM MgOAc, 2 mM DTT, 0.1% Tween 20, 0.1% casamino acids (binding buffer), and ribonuclease A was added (0.6 mg/ml) to prevent further translation. Aliquots of the translation product (45 μl) were mixed with 1.25 μg of control rabbit immunoglobulin, or 750 ng of affinity-purified rabbit anti-karyopherin α1 (hSRP1) or 750 ng of affinity-purified rabbit anti-karyopherin α2 (Rch1) (Nadler *et al.*, 1997), and were incubated for 1 h at 4°C in a total volume of 50 μl. Protein G Sepharose beads were then added (35 μl of a 50% slurry per sample) and the mix was incubated for an additional 30 min at 4°C. The precipitates were washed three times with binding buffer, and proteins were eluted with Laemmli sample buffer. Eluted proteins were resolved by 10–20% SDS–PAGE and were detected by fluorography.

Acknowledgements

We are grateful to N.Landau for the kind gift of the Vpr-expressing baculovirus, to S.Adam for p97 β karyopherin, to S.Nadler for Rch1 and antisera to Rch1 and hSrp1 α karyopherins, to A.Lamond for hSRP1α, to D.Görlich for human karyopherin β, to M.Rush for human Ran and to U.Grundmann for p10. Sheep polyclonal anti-MA serum (from M.Phelan) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. We would like to thank K.Manogue, M.Yamin, O.Haffar and the members of Bukrinsky lab for discussions and valuable suggestions on the manuscript. We are indebted to A.Cerami for his support. This work was supported in part by the NIH grants AI 33776 (M.B.), AI 36071 (L.R.) and by the funds from The Picower Institute. M.R. was supported in part by a fellowship from the Jane Coffin Childs Memorial Fund for Cancer Research. N.R. was supported by the grant RE 1228/2-1 from the Deutsche Forschungsgemeinschaft.

References

Adachi,A., Gendelman,H.E., Koenig,S., Folks,T., Willey,R., Rabson,A. and Martin,M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. *J. Virol.*, **59**, 284–291.

Adam,S.A. and Gerace,L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell*, **66**, 837–847.

Balliet,J.W., Kolson,D.L., Eiger,G., Kim,F.M., McGann,K.A., Srinivasan,A. and Collman,R. (1994) Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. *Virology*, **200**, 623–631.

Balotta,C., Lusso,P., Crowley,R., Gallo,R.C. and Franchini,G. (1993) Antisense phosphorothioate oligodeoxynucleotides targeted to the vpr gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. *J. Virol.*, **67**, 4409–4414.

Brown,P.O., Bowerman,B., Varmus,H.E. and Bishop,J.M. (1987) Correct integration of retroviral DNA *in vitro*. *Cell*, **49**, 347–356.

Brown,P.O., Bowerman,B., Varmus,H.E. and Bishop,J.M. (1989) Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. *Proc. Natl Acad. Sci. USA*, **86**, 2525–2529.

Brown,P.O. (1990) Integration of retroviral DNA. *Curr. Top. Microbiol. Immunol.*, **157**, 19–48.

Bukrinsky,M., Sharova,N. and Stevenson,M. (1993) Human immunodeficiency virus type 1 2-LTR circles reside in a nucleoprotein complex which is different from the preintegration complex. *J. Virol.*, **67**, 6863–6865.

Bukrinsky,M.I., Sharova,N., Dempsey,M.P., Stanwick,T.L., Bukrinskaya,A.G., Haggerty,S. and Stevenson,M. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl Acad. Sci. USA*, **89**, 6580–6584.

Bukrinsky,M.I *et al*. (1993a) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature*, **365**, 666–669.

Bukrinsky,M.I., Sharova,N., McDonald,T.L., Pushkarskaya,T., Tarpley,W.G. and Stevenson,M. (1993b) Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl Acad. Sci. USA*, **90**, 6125–6129.

Chi,N.C., Adam,E.J. and Adam,S.A. (1995) Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.*, **130**, 265–274.

Chi,N.C., Adam,E.J., Visser,G.D. and Adam,S.A. (1996) RanBP1 stabilizes the interaction of Ran with p97 nuclear protein import. *J. Cell Biol.*, **135**, 559–569.

Connor,R.I., Chen,B.K., Choe,S. and Landau,N.R. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology*, **206**, 935–944.

Dean,D.A. and Kasamatsu,H. (1994) Signal- and energy-dependent nuclear transport of SV40 Vp3 by isolated nuclei. Establishment of a filtration assay for nuclear protein import. *J. Biol. Chem.*, **269**, 4910–4916.

Di Marzio,P., Choe,S., Ebright,M., Knoblauch,R. and Landau,N.R. (1995) Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J. Virol.*, **69**, 7909–7916.

Dubrovsky,L., Ulrich,P., Nuovo,G.J., Manogue,K.R., Cerami,A. and Bukrinsky,M. (1995) Nuclear localization signal of HIV-1 as a novel target for therapeutic intervention. *Mol. Med.*, **1**, 217–230.

Dworetzky,S.I., Lanford,R.E. and Feldherr,C.M. (1988) The effects of variations in the number and sequence of targeting signals on nuclear uptake. *J. Cell Biol.*, **107**, 1279–1287.

Emerman,M., Bukrinsky,M. and Stevenson,M. (1994) HIV-1 infection of non-dividing cells. *Nature*, **369**, 107–108.

Fletcher,T.M *et al.* (1996) Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). *EMBO J.*, **15**, 6155–6165.

Folks,T.M. *et al.* (1986) Biological and biochemical characterization of a cloned Leu-3^{-} cell surviving infection with the acquired immune deficiency syndrome retrovirus. *J. Exp. Med.*, **164**, 280–290.

Fouchier,R.A., Meyer,B.E., Simon,J.H., Fischer,U. and Malim,M.H. (1997) HIV-1 infection of non-dividing cells: evidence that the N-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *EMBO J.*, **16**, 4531–4539.

Freed,E.O., Englund,G. and Martin,M.A. (1995) Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J. Virol.*, **69**, 3949–3954.

Gallay,P., Stitt,V., Mundy,C., Oettinger,M. and Trono,D. (1996) Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J. Virol.*, **70**, 1027–1032.

Gallay,P., Hope,T., Chin,D. and Trono,D. (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl Acad. Sci. USA*, **94**, 9825–9830.

Gorlich,D., Kraft,R., Kostka,S., Vogel,F., Hartmann,E., Laskey,R.A., Mattaj,I.W. and Izaurraide,E. (1996a) Importin provides a link between nuclear protein import and U snRNA export. *Cell*, **87**, 21–32.

Gorlich,D., Pante,N., Kutay,U., Aebi,U. and Bischoff,F.R. (1996b) Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.*, **15**, 5584–5594.

Gorlich,D., Kostka,H, Kraft,R., Dingwall,C., Laskey,R.A., Hartmann,E. and Prehn,S. (1995) Two different subunits of importin co-operate to recognise nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.*, **5**, 383–392.

Grundmann,U., Nerlich,C., Rein,T., Lottspeich,F. and Kupper,H.A. (1988) Isolation of cDNA coding for the placental protein 15 (PP15). *Nucleic Acids Res.*, **16**, 4721.

Heinzinger,N.K. *et al.* (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl Acad. Sci. USA*, **91**, 7311–7315.

Hu,T., Guan,T. and Gerace,L. (1996) Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J. Cell Biol.*, **134**, 589–601.

Imamoto,N., Tachibana,T., Matsubae,M. and Yoneda,Y. (1995) A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.*, **270**, 8559–8565.

Iovine,M.K., Watkins,J.L. and Wente,S.R. (1995) The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *J. Cell Biol.*, **131**, 1699–1713.

Jeoung,D.I., Chen,S., Windsor,J. and Pollack,R.E. (1991) Human major HSP70 protein complements the localization and functional defects of cytoplasmic mutant SV40 T antigen in Swiss 3T3 mouse fibroblast cells. *Genes Dev.*, **5**, 2235–2244.

Kalderon,D., Richardson,W.D., Markham,A.T. and Smith,A.E. (1984) Sequence requirements for nuclear location of simian virus 40 large T antigen. *Nature*, **311**, 33–38.

Kann,M., Bischof,A. and Gerlich,W.H. (1997) *In vitro* model for the nuclear transport of the Hepadnavirus genome. *J. Virol.*, **71**, 1310–1316.

Kraemer,D.M., Strambio-de-Castillia,C., Blobel,G. and Rout,M.P. (1995) The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. *J. Biol. Chem.*, **270**, 19017–19021.

Lewis,P., Hensel,M. and Emerman,M. (1992) Human immunodeficiency virus infection of cells arrested in the cell. *EMBO J.*, **11**, 3053–3058.

Lu,Y.L., Spearman,P. and Ratner,L. (1993) Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J. Virol.*, **67**, 6542–6550.

Mahalingam,S., Ayyavoo,V., Patel,M., Kieber-Emmons,T. and Weiner,D.B. (1997) Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. *J. Virol.*, **71**, 6339–6347.

Melchior,F., Paschal,B., Evans,J. and Gerace,L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.*, **123**, 1649–1659.

Miller,M.D., Farnet,C.M. and Bushman,F.D. (1997) Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J. Virol.*, **71**, 5382–5390.

Moore,M.S. and Blobel,G. (1992) The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell*, **69**, 939–950.

Moore,M.S. and Blobel,G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, **365**, 661–663.

Moroianu,J., Blobel,G. and Radu,A. (1995a) Previously identified protein of uncertain function is karyopherin alpha and together with karyopherin beta docks import substrate at nuclear pore complexes. *Proc. Natl Acad. Sci. USA*, **92**, 2008–2011.

Moroianu,J., Hijikata,M., Blobel,G. and Radu,A. (1995b) Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 6532–6536.

Moroianu,J., Blobel,G. and Radu,A. (1996) The binding site of karyopherin alpha for karyopherin beta overlaps with a nuclear localization sequence. *Proc. Natl Acad. Sci. USA*, **93**, 6572–6576.

Nadler,S.G., Tritschler,D., Haffar,O.K., Blake,J., Bruce,A.G. and Cleaveland,J.S. (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. *J. Biol. Chem.*, **272**, 4310–4315.

Okuno,Y., Imamoto,N. and Yoneda,Y. (1993) 70-kDa heat-shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport *in vitro*. *Exp. Cell Res.*, **206**, 134–142.

Popov,S. *et al.* (1996) Critical role of reverse transcriptase in the inhibitory mechanism of CNI-H0294 on HIV-1 nuclear translocation. *Proc. Natl Acad. Sci. USA*, **93**, 11859–11864.

Radu,A., Blobel,G. and Moore,M.S. (1995) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 1769–1773.

Ren,M. *et al.* (1995) Separate domains of the Ran GTPase interact with different factors to regulate nuclear protein import and RNA processing. *Mol. Cell Biol.*, **15**, 2117–2124.

Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors and nucleoporins. *Cell*, **83**, 683–692.

Schmidtmayerova,H., Nuovo,G.J. and Bukrinsky,M. (1997) Cell proliferation is not required for productive HIV-1 infection of macrophages. *Virology*, **232**, 379–384.

Shi,Y. and Thomas,J.O. (1992) The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell Biol.*, **12**, 2186–2192.

Shulga,N., Roberts,P., Gu,Z., Spitz,L., Tabb,M.M., Nomura,M. and Goldfarb,D.S. (1996) *In vivo* nuclear transport kinetics in *Saccharomyces cerevisiae*: a role for heat shock protein 70 during targeting and translocation. *J. Cell Biol.*, **135**, 329–339.

Varmus,H. and Swanstrom,R. (1985) Replication of retroviruses. In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 369–512.

von Schwedler,U., Kornbluth,R.S. and Trono,D. (1994) The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc. Natl Acad. Sci. USA*, **91**, 6992–6996.

Weis,K., Mattaj,I.W. and Lamond,A.I. (1995) Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. *Science*, **268**, 1049–1053.

Westervelt,P., Henkel,T., Trowbridge,D.B., Orenstein,J., Heuser,J., Gendelman,H.E. and Ratner,L. (1992a) Dual regulation of silent and productive infection in monocytes by distinct human immunodeficiency virus type 1 determinants. *J. Virol.*, **66**, 3925–3931.

Westervelt,P. *et al.* (1992b) Macrophage tropism determinants of human immunodeficiency virus type 1 *in vivo*. *J. Virol.*, **66**, 2577–2582.

Yao,X.J., Subbramanian,R.A., Rougeau,N., Boisvert,F., Bergeron,D. and Cohen,E.A. (1995) Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. *J. Virol.*, **69**, 7032–7044.

Zhao,L.J., Mukherjee,S. and Narayan,O. (1994) Biochemical mechanism of HIV-I Vpr function. Specific interaction with a cellular protein. *J. Biol. Chem.*, **269**, 15577–15582.

Received November 21, 1997; revised January 7, 1998; accepted January 8, 1998