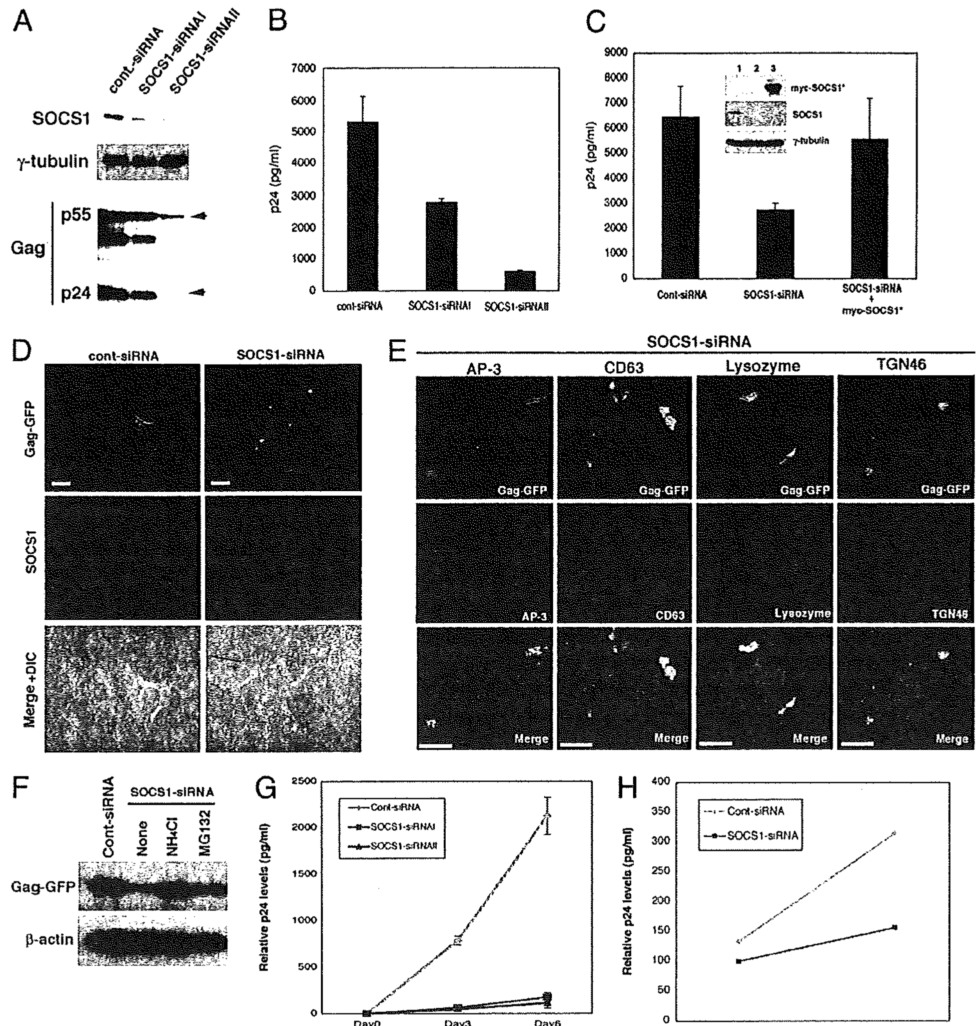

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, cell lysates were subjected to immunoblotting analysis with the indicated antibodies (*A*). Cell supernatants were then subjected to ELISA analysis of p24 levels (*B*). (*C*) 293T cells were transfected with pNL4-3 and cotransfected with control-siRNA, SOCS1-siRNAI alone, or SOCS1-siRNAI plus siRNA-resistant myc-SOCS1 (myc-SOCS1*). After 48 h, cell supernatants were collected and subjected to p24 ELISA. (*Inset*) Immunoblots of the cell lysates. (*D*) HeLa cells were transfected with control or SOCS1-specific siRNA and cotransfected with GFP-Gag. At 48 h after transfection, the cells were subjected to confocal microscopy. (*E*) HeLa cells were transfected with Gag-GFP and SOCS1-siRNA constructs for 48 h. Cells were then fixed and subjected to immunofluorescent analysis with indicated antibodies followed by DAPI staining. (Scale bars: 10 mm.) (*F*) HeLa cells were transfected with Gag-GFP and cotransfected with either control-siRNA or SOCS1-siRNA. After 36 h, the cells were treated with a mock solution, 10 mM NH_4Cl or 10 μM MG132 for another 16 h. Cell were then harvested and subjected to immunoblotting analysis with anti-GFP or anti-β-actin antibodies. (*G*) Jurkat cells were infected with a retroviral vector encoding control (Cont) or two different SOCS1-specific siRNAs (I or II). After selection with puromycin, the cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points. (*H*) Human primary CD4 T cells were separated from healthy donors and infected with lentivirus vectors encoding either control- or SOCS1-siRNAI. The cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points.

this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3*C*), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3*D*). These results again indicate that SOCS1 efficiently enhances the trafficking of newly synthesized Gag protein to PM.

The Targeted Disruption of SOCS1 Inhibits Gag Trafficking and HIV-1 Particle Production. To delineate further the role of SOCS1 in the trafficking of Gag and in subsequent HIV-1 particle production, we depleted cellular SOCS1 by siRNA. The significant depletion of SOCS1 expression by two different SOCS1-specific siRNA constructs was confirmed by immunoblotting analysis (Fig. 4 *A* and *B*). Significantly, in cells cotransfected with pNL4-3 and SOCS1-specific siRNAs, both HIV-1 particle release and the levels of intracellular Gag protein are significantly decreased compared with the control cells (Fig. 4 *A* and *B*). Furthermore, the effects of SOCS1-siRNA on the inhibition of HIV-1 particle production was diminished by reexpression with a codon-optimized SOCS1 construct that is resistant to these siRNAs (Fig. 4*C*), indicating that the SOCS1 siRNA suppression of HIV-1 particle production depends on the availability of endogenous SOCS1.

Consistent with these observations, immunofluorescent analysis further revealed that the expression of SOCS1-siRNA dramatically inhibits Gag trafficking such that Gag proteins accumulate in the perinuclear regions as large solid aggregates, as has been reported (20) (Fig. 4*D*). This finding indicates that SOCS1 plays an essential role in the Gag trafficking from perinuclear clusters to PM. Interestingly, these discrete perinuclear clusters of Gag were found to colocalize with lysosome markers, lysozyme, and partly with AP-3, but neither with the late endosome MVB marker CD63 nor the *trans*-Golgi marker TGN46, indicating that Gag is targeted for degradation by lysosomes when the function of SOCS1 is inhibited (Fig. 4*E*). In support of this notion, the levels of intracellular Gag were found to be significantly increased by treatment with a lysosome inhibitor NH_4Cl but not by a proteasome inhibitor MG132 in SOCS1-siRNA cells (Fig. 4*F*), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

We next addressed whether targeted SOCS1 inhibition would affect HIV-1 particle production in human T cells. The effect of SOCS1 depletion was clearly evident in both HIV-1_{NL4-3}-infected

Jurkat cells and human primary CD4+ T cells, which demonstrated pronounced decreases in virus particle production in SOCS1-siRNA-expressed cells compared with the controls (Fig. 4 *G* and *H*). These results together indicate that the specific inhibition of SOCS1 suppresses the optimal trafficking of Gag to PM, resulting in the degradation of Gag in lysosomes, which in turn leads to the efficient and reproducible inhibition of HIV-1 particle production in various types of human cells.

Discussion

In this work, we report that SOCS1 is an inducible host factor during HIV-1 infection and plays a key role in the late stages of the viral replication pathway via an IFN-independent mechanism (SI Fig. 6). These results represent evidence that SOCS1 is a potent host factor that facilitates HIV-1 particle production via posttranscriptional mechanisms.

SOCS1 has been shown to be a suppressor of several cytokine signaling pathways, and like all SOCS family members it has a central SH2 domain and a conserved C-terminal domain known as the SOCS box (21, 22). Structure–function analyses have further demonstrated that the SOCS1 SH2 domain is required for the efficient binding of its substrates (23, 24). Indeed, our current analyses have also revealed that the SH2 domain of SOCS1 is required for its interaction with the HIV-1 Gag protein. We have shown from our present data that the SOCS box is also required for SOCS1 to function during HIV-1 particle production.

The SOCS box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity (21, 25). Biochemical binding studies have shown that the SOCS box of SOCS1 interacts with the elongin BC complex, a component of the ubiquitin/proteasome pathway that forms an E3 ligase with Cul2 (or Cul5) and Rbx-1 (21, 26, 27). We show from our current experiments that the SOCS box is required for HIV-1 particle production, indicating the involvement of the ubiquitin/proteasome pathway. However, it is still unknown whether SOCS1 promotes the ubiquitination of Gag and, if so, whether the mono- or poly-ubiquitination of Gag would affect its trafficking and protein stability. Further studies will be necessary to clarify the biological significance of Gag ubiquitination.

Perlman and Resh (20) recently reported that newly synthesized Gag first appears to be diffusely distributed in the cytoplasm, accumulates in perinuclear clusters, passes transiently through a MVB-like compartment, and then traffics to PM. Consistent with these observations, our current work also shows that Gag is accumulated at perinuclear clusters as solid aggregates when its targeting to PM is impaired because of the SOCS1 inhibition.

Another aspect of SOCS1 function during HIV-1 infection was proposed recently. Song *et al.* (28) reported that SOCS1-silenced dendritic cells broadly induce the enhancement of HIV-1 Env-specific CD8+ cytotoxic T lymphocytes and CD4+ T helper cells as well as an antibody response. The induction of the SOCS1 gene in HIV-1 infected cells might therefore disrupt a specific intracellular immune response to HIV-1 in infected host cells.

Based on the strong evidence that we present in our current work that SOCS1 positively regulates the late stages of HIV replication, we conclude that SOCS1 is likely to be a valuable therapeutic target not only for future treatments of AIDS and related diseases, but also for a postexposure prophylaxis against disease in HIV-1-infected individuals.

Materials and Methods

Antibodies and Fluorescent Reagents. Antibodies and fluorescent reagents were obtained from the following sources. Anti-CD63, anti-AP-3, anti-myc (A-14), and anti-SOCS1 (H-93) were from Santa Cruz Biotechnology. Anti-SOCS1 was from Zymed Laboratories. Anti-FLAG (M2) and anti-HA (12CA5) were from Sigma and Roche Diagnostics, respectively. Anti-HIV-p24 (Dako; Cytomation), anti-STAT1, and anti-phospho-STAT1 (Y701) were from BD Transduction Laboratories. Sheep polyclonal anti-TGN46 was from GeneTex.

Plasmid Constructs. Expression constructs for SOCS1 have been described in ref. 29. GST fusion constructs with specific regions derived from the codon-optimized *gag* were generated (MA, CA, NC, p6, Δp6, full-length Gag) by cloning into pGEX-2T (GE Healthcare Bio-Sciences) as described in ref. 30. For retrovirus-mediated siRNA expression, pSUPER.retro.puro vector was digested, as described in ref. 31, with the following sequences: SOCS1-siRNAI, TCGAGCTGCTGGAGCACTA; SOCS1-siRNAII, GGCCAGAACCTTCCTCCTCTT; control siRNA, TCGTATGTTGTGTGGAATT.

Electron Microscopy. Transfected 293T cells were fixed with 2.5% glutaraldehyde and subjected to TEM, as described (14, 32).

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Role of Vpr in HIV-1 Nuclear Import: Therapeutic Implications

Yoko Aida* and Go Matsuda

Viral Infectious Diseases Unit, RIKEN, Wako, Saitama, Japan

Abstract: The replication of human immunodeficiency virus type 1 (HIV-1) in non-dividing cells, such as terminally differentiated macrophages, critically depends on the import of the viral pre-integration complex (PIC) into the nucleus. Vpr, one of the accessory gene products of HIV-1, plays a key regulatory role in PIC nuclear import in macrophages, although its role in the PIC entry mechanism remains to be clarified. Here, we summarize what is currently known about the nuclear-entry step of HIV-1 replication, mainly focusing on how Vpr functions as the main regulator of HIV-1 nuclear import and how it could facilitate the development of novel inhibitors of this process.

Keywords: HIV-1Vpr, nuclear import, pre-integration complex (PIC), Importin α, nucleoporins, non-dividing cells, nuclear pore complex (NPC).

INTRODUCTION

DNA viruses and some RNA viruses must access the nucleus to replicate [1, 2]. The interior of the nucleus is separated from the cytoplasm by a double-layer membrane contiguous with the ER and called the nuclear envelope [3]. This nuclear envelope is comprised of two lipid bilayers, the outer and inner nuclear membranes [4]. These membranes are separated by a lumen and joined at nuclear pore complexes (NPCs) [5] that serve as gates for traffic crossing the nuclear envelope. Entrance into and exit from the nucleus occurs *via* these NPCs, which are > 60 MD macromolecular structures that form channels spanning the nuclear envelope [6, 7]. Each NPC is equipped to facilitate both import and export of proteins and RNAs [8].

The movement of ions, metabolites, and other small molecules through the NPC occurs *via* passive diffusion, but the translocation of cargoes larger than ~40 kD generally requires specific signals known as nuclear localization signals (NLSs) [9]. The nuclear import of basic NLS-bearing proteins is mediated by specific soluble factors, including importin-α (Impα) [10], importin-β (Impβ) [11], small GTPase Ran/TC4 [12], and NTF2 [13]. Impα functions as an adaptor molecule, binding Impβ *via* its amino-terminally located Impβ-binding (IBB) domain and binding an NLS-bearing protein *via* its two central region–located NLS-binding sites [14, 15]. Impβ is the transport receptor that carries the Impα–NLS complex from the cytoplasm to the nuclear side of the NPC. Once the heterotrimer consisting of Impα, Impβ, and the NLS-bearing protein reaches the nuclear face of the NPC, the GTP-bound form of Ran binds directly to Impβ, releasing Impα and the NLS-bearing protein into the nucleoplasm. Ran, which is found in its GDP-bound form in the cytoplasm and in its GTP-bound form in the nucleus, is a major determinant of the directionality of transport across the nuclear membrane. However, there are many additional pathways that mediate nuclear import; multiple classes of Impβ–like molecules (i.e., tranportin or importin 7 [Imp7]) present in the cell carry a variety of transport cargoes into the nucleus [9].

In dividing cells, the breaking and re-forming of the nuclear envelope during each cycle of cell division allows the straightforward exchange of material between the nuclear and cytoplasmic compartments. Retroviruses such as Moloney murine leukemia virus require cell division to replicate, and solve the nuclear entry problem by waiting for the nuclear envelope to break down during mitosis [16-19]. In contrast, HIV-1 can infect both dividing and non-dividing cells [19-23]. Importantly, non-dividing cells, such as terminally differentiated macrophages, are particularly important targets for viral replication during the initial stages of infection, since primary infection of these cell populations helps to establish the viral reservoirs crucial for the subsequent spread of virus to lymphoid organs and T-helper lymphocytes [24, 25]. The ability of HIV-1 to replicate in non-dividing cells depends on the active nuclear import of a large nucleoprotein complex called pre-integration complex (PIC), which is a process requiring energy [22]. Understanding how HIV-1 can infect terminally differentiated, non-dividing cells has proven a very complex and controversial problem. It is, however, a problem worth investigating, for it is central to HIV-1 transmission and AIDS pathogenesis.

In this review, we summarize what is currently known about the nuclear-entry step of retroviral replication. The emerging model depicts the HIV-1 accessory protein Vpr as the main regulator of HIV-1 nuclear import, and indicates that Vpr facilitates development of novel inhibitors of the nuclear import process.

FORMATION AND TRANSPORT OF HIV-1 PIC

HIV-1 has the unique ability to replicate efficiently in non-dividing cells, such as macrophages. Mitosis-independent nuclear import is required to transform into the large nucleoprotein complex PIC. PIC is formed in the early stages of infection, as shown in Fig. (**1**). The HIV-1 virion attaches to a target cell and fuses its envelope to the plasma membrane. Next, the core containing viral genomic RNA is released into the cytoplasm. Reverse transcription of viral

*Address correspondence to this author at the Viral Infectious Diseases Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; Tel: +81-48-462-4408; Fax: +81-48-462-4399; E-mail: aida@riken.jp

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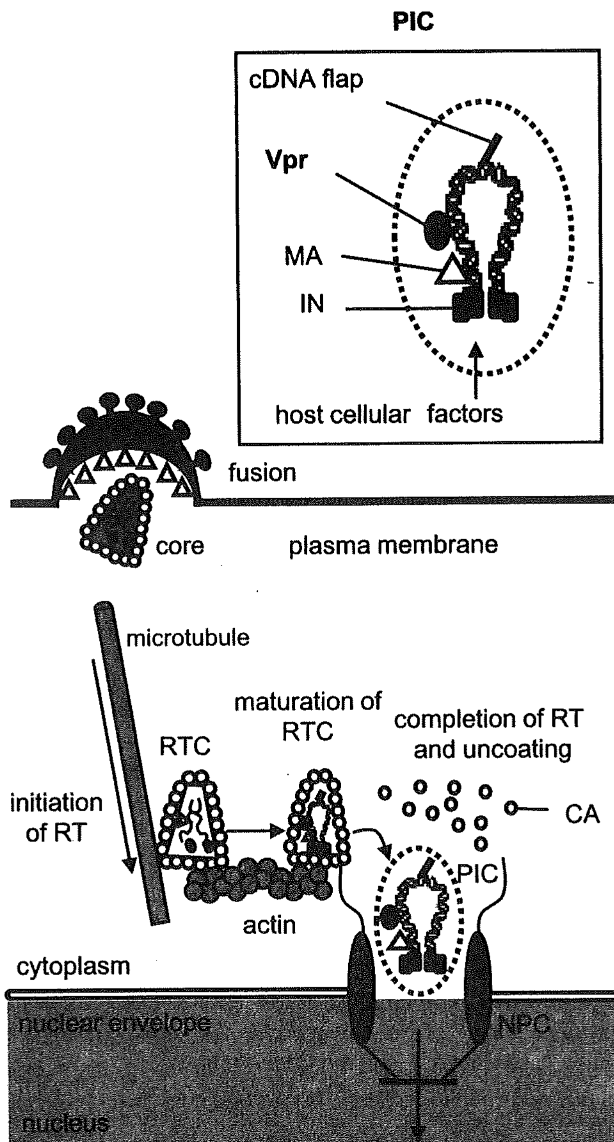

Fig. (1). HIV-1 nuclear import model. After cellular infection, the viral core containing genomic RNA is released into the cytoplasm. The core moves along the microtubules and actin filaments to the nearby nuclear pore complex (NPC). Following reverse transcription and in a central DNA (cDNA) flap–dependent manner, capsid (CA) is uncoated from a matured reverse transcription complex (RTC). Then, pre-integration complex (PIC) containing viral complementary DNA–associating viral proteins such as integrase (IN), matrix (MA) or Vpr, can enter into the nucleus together with cellular factors.

RNA partially begins in the capsid shell known as the reverse transcription complex (RTC) [26]. The RTC moves to the nuclear envelope along the microtubules [26] and actin filaments [27]. The RTC then docks at the NPC and reverse transcription is completed, following which uncoating occurs in a central DNA (cDNA) flap–dependent manner [28]. Early studies suggested that uncoating occurs immediately after entry into the cytoplasm, since the capsid (CA) is barely detectable, having readily dissociated from PIC during sample preparation [29]. However, more recent reports have indicated that CA must dissociate from PIC in order for un-coating to occur after the completion of reverse transcription at the NPC [28]. However, the relationship between the speed of un-coating and nuclear import remains unclear [30]. Finally, matured RTC recruits cellular factors, and PIC is transformed and enters the nucleus.

As shown in Fig. (**1**), PIC contains viral and cellular proteins in addition to viral DNA. To date, various components have been reported and their involvement remains controversial; these include reverse transcriptase (RT), Integrase (IN), Matrix (MA), Nucleocapsid (NC), and Vpr [29, 31-34]. It has also been proposed that NC and RT prefer viral RNA to DNA and are dissociated from PIC after the completion of reverse transcription [34-36].

NLS AND THE NUCLEAR IMPORT OF HIV-1 PIC

Once the retroviral nucleoprotein complex reaches the nucleus, it must cross the nuclear envelope to integrate into the chromosomal DNA. How does HIV-1 PIC cross the intact nuclear envelope during interphase in non-dividing cells? Theoretically, molecules of up to 9 nm in diameter can pass through the NPC by passive diffusion [37]. However, the retroviral PIC is too large for passive transport; the HIV-1 PIC is estimated to be > 56 nm in diameter [33, 35], which is larger than the 39 nm maximum dimension for karyophilic macromolecules passing through the NPC [38]. Although the underlying molecular mechanisms of PIC nuclear import are unknown, there is enough evidence to discuss several possibilities (Fig. **2**).

The first possibility resides in the uncoating process. Interestingly, a small PIC (~80S) is detected in the nucleus, while a larger PIC (~100-350S) is detected in the cytoplasm [29]. Thus, significant shedding of components and/or major conformational changes must occur to reduce its size, which indicates that CA dissociates from RTC. In fact, HIV-1 appears to shed its capsid shell during infection, presumably after reverse transcription is completed at the nuclear pore [28]. Therefore, the uncoating step is necessary for efficient nuclear import.

Second, although viral capsid uncoating is likely a prerequisite for nuclear import, specific signals and import factors also likely direct the intracellular trafficking of PIC, which contains IN, Vpr, and MA, in addition to cDNA flap [29, 31, 34, 39]. Importantly, MA, Vpr, and IN possess one or several NLSs and are associated with viral nucleic acids after infection. These viral factors have also been implicated in PIC nuclear import *via* interaction with NLS receptors (i.e., importins or karyopherins); however, the contribution of these interactions to HIV-1 replication in non-dividing cells is controversial. For example, IN can bind to Impα, an adaptor protein between NLS-containing proteins and Impβ [40-42], and Impβ, which ferries into the nucleus the Impα/NLS complex or proteins containing basic stretches [41, 42]. Along with Impβ, IN also binds Imp7, an import receptor for ribosomal proteins and histone H1 [41]. Finally, IN binds transportin, which transports hnRNP A1 [41]. Impβ, Imp7, and the Impα/Impβ and Impβ/Imp7 heterodimers have been shown to stimulate nuclear accumulation of IN [41]. Vpr can also bind Impα [43-45]. Following this binding, Vpr is transported into the nucleus by an interaction with Impα alone [45, 46], but not with the Impα/Impβ heterodimer [43, 47]. Furthermore, the N-terminal NLS region

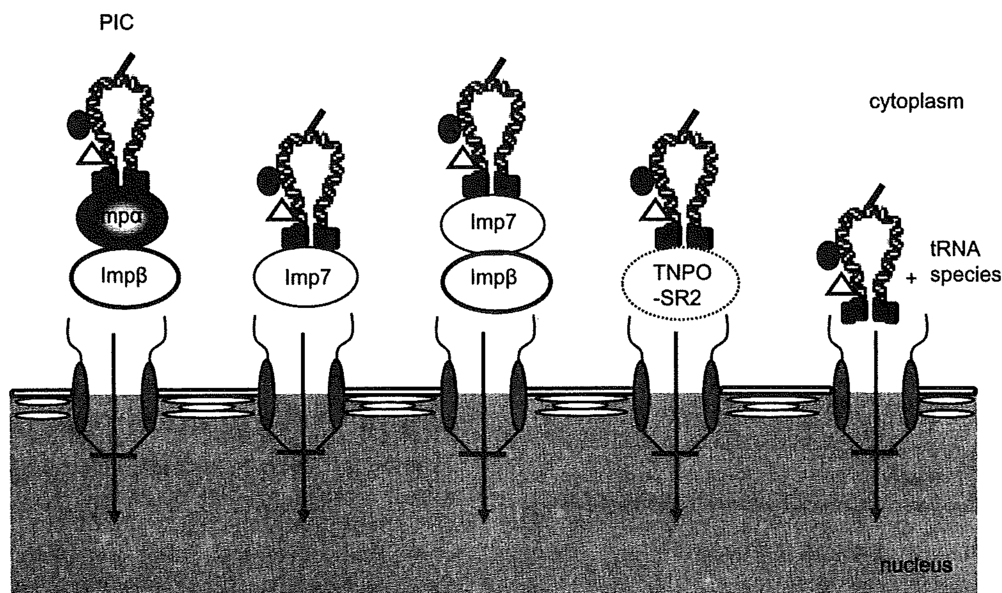

Fig. (2). Model of HIV-1 PIC nuclear import machinery. The Impα/Impβ heterodimer, Imp7, the Imp7/Impβ heterodimer, TNPO-SR2 or tRNA species promote the nuclear import of HIV-1 PIC.

of MA also interacts with Impα, supporting a role for MA in nuclear import [48, 49]. Thus, we hypothesize that IN, MA and Vpr work either sequentially or synergistically to regulate PIC nuclear import. However, nuclear import assays with purified PIC clearly show that Imp7 and the Imp7/Impβ heterodimer, but neither the Impα/Impβ heterodimer nor transportin [41], stimulate PIC nuclear import. Likewise, a recent study indicates that transportin-SR2, which transports serine/arginine-rich proteins (SRs), mediates PIC nuclear import and therefore facilitates HIV-1 infection [50].

Third, a new player, tRNA, which mediates translation in the cytoplasm, may facilitate PIC nuclear import [51]. In particular, some tRNA species lacking the 3' CCA end are critical for nuclear import. Furthermore, these tRNA species are incorporated into budding viral particles, and tRNA has been shown to travel in a retrograde direction from the cytoplasm to the nucleus [52, 53].

NEW INSIGHTS INTO VPR-MEDIATED NUCLEAR IMPORT IN HIV-1 REPLICATION

As mentioned above, the exact composition of the HIV-1 PIC is still open to question, although many recent studies have confirmed that Vpr is an integral component of the HIV-1 PIC [1, 54-57]. The HIV-1 accessory protein Vpr, a small (14-kDa) nuclear protein of 96 amino acids, is a virion-associated protein [54, 58-60]. Vpr increases HIV-1 replication in non-dividing macrophages, possibly by facilitating and promoting PIC nuclear uptake together with other viral components [40, 48, 61] and by engaging in direct or indirect interactions with cellular factors [43, 44, 48, 60, 62-65]. Mutant viruses that lack an intact *vpr* gene do not replicate as well as wild-type viruses, particularly in non-dividing cells; for instance in macrophages, decreases in replication of ~10 fold have been observed for such mutants [60, 62, 63]. While some reports support such observations [44, 66, 67], others have not [68-70], and the contribution of Vpr to the productive infection of HIV-1 in non-dividing cells remains controversial.

Vpr Nuclear Import Signals

Both MA and IN have functional NLSs that resemble the canonical NLS of the simian virus (SV) 40 T-antigen [40, 48, 71]. In contrast, despite the lack of an identifiable canonical NLS, Vpr displays karyophilic properties and is not only rapidly targeted to host-cell nuclei after infection [59], but is also predominantly localized in the nucleus and nuclear envelope when expressed on its own [44, 45, 67, 72]. Studies have clearly confirmed that Vpr nuclear import is a signaling-mediated process that proceeds through the NPC [45, 47]. Consistent with the absence of a classical import signal, Vpr nuclear import is unaffected by the excess addition a peptide corresponding to NLS of SV40 large T antigen or the Impα IBB domain [40, 47]. It is also unaffected by the M9 signal sequence [47], which is located in hnRNP A1 and is associated with transportin-mediated nuclear import [73, 74]. Furthermore, a dominant-negative mutant form of Ran, RanQ69L, that potently suppresses nuclear import [75, 76], does not inhibit Vpr nuclear import [47], suggesting that Vpr does not require the Ran-mediated import pathway. Moreover, our *in vitro* transport assay showed that Vpr nuclear import is mediated by Impα alone [45]. These observations strongly suggest that that Vpr-mediated transport occurs *via* non-classical targeting signals.

What do we know about the NLS of Vpr? NMR structural analyses reveal that the full-length Vpr forms three alpha helices (αH1, αH2, and αH3) surrounding a hydrophobic core [77]. The N-terminal domain is flexible and negatively charged, and flanks the helices. The C-terminal domain is also flexible, is positively charged, and is rich in arginine residues [77]. The region between residues 17 and 74 was identified as a bona fide NLS that consists of two independent functional regions, αH1 and αH3 [45, 78]. Interestingly, each of these regions plays two different roles

and is indispensable for the nuclear entry of Vpr. Similar results have been reported by Mahalingam *et al.* [79]. In contrast, the Vpr C-terminal domain, which most closely resembles a classical NLS, is heavily involved in Vpr nuclear localization [47, 80, 81]. However, contrary results have also been reported, as this region can be deleted without impairing nuclear localization [82]. Moreover, a peptide derived from this region did not function as an active NLS [83]. Our results clearly indicate that the Vpr C-terminal domain is not absolutely required for nuclear localization [78]. The C81 mutant (Vpr with a C-terminal 15-aa deletion) displays an immunofluorescence staining pattern similar to that of wild-type Vpr. Furthermore, substitutions within the αH1 and αH3 domains of Vpr with an intact C-terminal domain result in almost no nuclear staining [78].

Vpr-Mediated Nuclear Import

Although the molecular mechanisms by which Vpr cross the nuclear envelope remain poorly understood, Fig. (**3**) summarizes the several hypotheses proposed for the mode of action of Vpr in HIV-1 nuclear import.

First, numerous investigations of the subcellular localization of Vpr [44, 59, 72, 78] have suggested that since the molecular mass of Vpr is less than the 40 kD size limit for passive diffusion of proteins through the NPC, Vpr may cross the nuclear envelope by passive diffusion and accumulate in the nucleus (Fig. **3A**).

Second, each NPC consists of 50 to 100 distinct nuclear pore proteins, named nucleoporins [7], and it is possible that Vpr enters the nucleus by interacting with these proteins (Fig. **3B**). This model is based on three separate observations. First, Vpr expressed without other viral proteins localizes in the nucleus, but a significant fraction is anchored at the nuclear envelope and can be visualized as a nuclear rim using fluorescence microscopy [44, 63, 78, 84]. Second, confocal microscopy and fluorescence photobleaching of COS-7 cells expressing Vpr fused to green fluorescent protein (GFP) at its C-terminus showed that Vpr-GFP associates dynamically with the nuclear envelope and exchanges with soluble Vpr-GFP and/or Vpr-GFP associated with adjacent areas of the nuclear envelope [65]. Third, in an *in vitro* nuclear import assay with digitonin-permeabilized cells in which Vpr was fused to GFP and glutathione S-transferase, Vpr targeted to the perinuclear region without requiring any soluble factors [45]. Finally, Vpr can, in fact, interact with components of the NPC [44, 63, 64]. A specific subset of nucleoporins contain phenylalanine–glycine (FG) or FxFG peptide repeats that constitute most of the filamentous structures emanating from both sides of the NPC and that provide docking sites for various transport factors [85]. Yeast two-hybrid and *in vitro* binding studies have shown that HIV-1 Vpr can bind to the FG-rich region of several nucleoporins, including human p54 and p58 Nups, yeast NUP1P and Nsp1p, and rodent POM121 [44, 63, 64]. A direct interaction of HIV-1 Vpr with the human CG1 nucleoporin was more recently reported [65, 86]. The interaction between Vpr and the NPC is crucial for Vpr nuclear import, since Vpr mutants, whose perinuclear localization is barely detectable, cannot be imported into the nucleus [45, 86]. Although the role of Vpr at the NPC is unclear, this Vpr localization may indicate that virion-associated Vpr might mediate PIC bind-

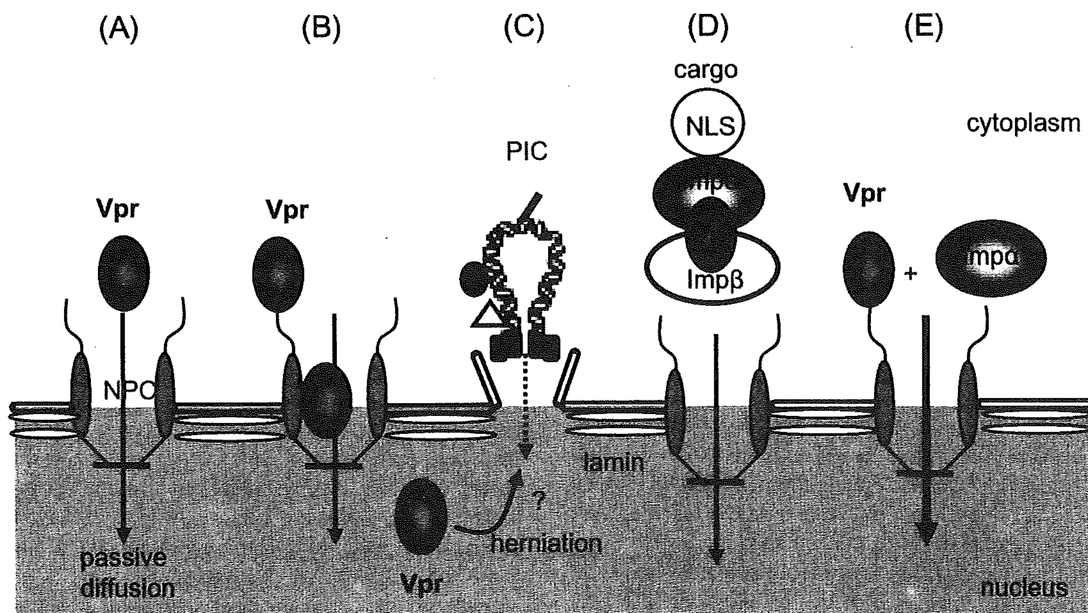

Fig. (3). Model of Vpr-mediated nuclear import. Although the role of Vpr is still controversial, there are several possible mechanisms that Vpr could use to cross the nuclear envelope. These are (**A**) Vpr could cross the nuclear envelope by passive diffusion and accumulate in the nucleus because it is small enough to pass through the NPC; (**B**) Vpr could enter the nucleus by interacting with nucleoporins, which make up the nuclear pore complex (NPC); (**C**) Vpr could enter the nucleus through the NPC of host cells and then induce transient, local herniations and disruptions of the lamina architecture in the nuclear envelope. This would allow the PIC to enter the nucleus from the nuclear envelope herniations disrupted by Vpr; (**D**) Vpr binding to Impα could stimulate subsequent nuclear import of the cargo by increasing the affinity of Impα and NLS-containing proteins; (**E**) The nuclear import of Vpr could be accelerated by Impα alone, without the intervention of the classical Impβ-dependent transport system.

ing to the nuclear pore after viral uncoating. This model is strongly supported by the finding that Vpr facilitates the docking of HIV-1 PICs by stabilizing the association between the FxFG repeat–containing nucleoporins and the import complex of Impα, Impβ and cargo [64]. Alternatively, it is also possible that, by interacting with nucleoporins, Vpr causes misassembly of the NPC, leading to alterations of the nuclear envelope architecture.

Third possibility has been raised following the observation that Vpr enters the nucleus through the NPC of host cells and then induces transient, local herniations and disruptions of the lamina architecture in the nuclear envelope [87, 88] (Fig. **3C**). This process might locally open the gate for the nuclear entry of PIC. Although there is no evidence yet that PICs pass through such localized regions in non-dividing cells, this finding supports previous studies showing that a disrupted nuclear-lamina structure coincides with herpes simplex virus infection [89].

Fourth, Vpr binds to Impα and this binding stimulates subsequent nuclear import of the cargo by increasing the affinity of Impα and NLS-containing proteins (Fig. **3D**). Indeed, an *in vitro* analysis using digitonin-permeabilized HeLa cells revealed that the addition of recombinant Vpr stimulates the nuclear import of bovine serum albumin conjugated to an SV40 T-antigen–NLS peptide sequence [43]. In addition, Vpr also regulates the nuclear import of HIV-1 PIC, by binding to Impα and increasing its affinity for viral NLSs, including the NLS of MA [43].

A fifth possibility is postulated from our discovery of a novel nuclear import mechanism for Vpr. We found that Vpr can target first to the NPC by interacting with the αH3 region, without requiring any soluble factors, and then enter the nucleus by Impα alone, in an Impβ-independent manner that involves the αH1 region [45, 46] (Fig. **3E**). Jenkins and colleagues [47] previously showed that the nuclear import of Vpr *in vitro* proceeds independently of protein factors provided by a rabbit reticulocyte lysate, as well as independently of exogenously added nucleoside triphosphates. These characteristics of Vpr do not coincide with those of NLS- or M9-binding proteins [47], suggesting that as yet unidentified cellular pathways and/or factors are involved in the import process. Therefore, we attempted to identify the factors necessary for Vpr nuclear entry by using an *in vitro* transport assay [45], and found that Vpr transport is mediated by Impα alone, without the intervention of Impβ, Impα/Impβ heterodimers, transportin, RanGDP, or NTF2. Moreover, the Impα derivative ΔIBB, which cannot bind Impβ, could also promote Vpr nuclear import, confirming that Vpr is transported by Impα without utilizing the classical Impβ-dependent transport system [46]. Consist with this novel nuclear import, Impα alone can also escort Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) into the nucleus [90]. Moreover, Vpr interacts with the C-terminal region of Impα, which overlaps with the binding region for CAS, the nuclear export factor of Impα, and this interaction is indispensable for nuclear entry of Vpr [45, 46]. This nuclear import of Vpr is promoted by all three major isoforms of Impα. Interestingly, similar to the case for Vpr, the nuclear transport of CaMKIV requires the C-terminal region of Impα and is promoted by all three major isoforms of Impα. In contrast, Bukrinsky and colleagues reported that Vpr associates with the N-terminal region of Impα, which overlaps with the IBB domain of Impα and differs from the classical NLS cargo binding site. This interaction increases the affinity of Impα for basic-type NLSs, including that of MA [64, 91]. In agreement with the forth model as mentioned above, our laboratory has also indicated that while the IBB domain of Impα binds strongly to Vpr, this binding is not essential for nuclear entry of Vpr itself [45].

It appears, therefore, that nuclear import of the HIV-1 PIC is controlled by Vpr through several pathways, which are mediated by interaction with Impα and nucleoporins, as mentioned above. Many questions, however, remain unanswered regarding Vpr and its nuclear entry pathways as well as its effects on the nuclear envelope. An important issue now will be to define the detailed mechanisms through which Vpr contributes to PIC nuclear entry in non-dividing cells, such as macrophages.

Importance of Impα in Vpr Nuclear Import in Macrophages

Monocytes and macrophages are major targets of HIV-1 and serve as viral reservoirs [92]. Although the extents of reverse transcription are similar in both cell types after infection with an HIV-based vector, nuclear entry is blocked in monocytes [93]. One key difference between these two cell types resides at the level of the Impα expression. In fact, immature monocytes express Impα at low levels, whereas the expression of the three major Impα isoforms markedly increases when moncytes differentiate into macrophages [46]. Interestingly, Vpr nuclear import is strongly promoted by the addition of a macrophage cytoplasmic extract but not by a monocyte cytoplasmic extract, and nuclear import activity is lost when Impα is depleted from the macrophage cytoplasmic extract [46], suggesting that Impα expression is required for Vpr nuclear import. This requirement for Impα in Vpr nuclear import in macrophages has been confirmed by showing that depletion of Impα from cytoplasmic extracts of macrophages prevents import. In addition, *in vitro* nuclear transport assays have demonstrated that siRNA depletion of Impα from cytoplasmic extracts of HeLa cells markedly decreases Vpr nuclear import. Moreover, experimental infection with a virus encoding a Vpr mutant protein that cannot bind to Impα showed that the expression of Impα is essential for viral replication in macrophages, and, in addition, that the interaction between Impα and the αH1 region of Vpr is indispensable for not only Vpr nuclear import but also for HIV-1 replication in macrophages [46]. There is a good possibility that Vpr nuclear import is inefficient in monocytes, which express Impα at a low level, and that therefore the PIC cannot enter the nucleus of these cells. The low level of Impα in monocytes may also be the cause of the inefficient nuclear import of MA and IN, which utilize the classical Impα/Impβ-dependent nuclear import pathway [48], as well as the cause of the inefficient nuclear import of the cargo by decreasing the affinity of Impα and NLS-containing proteins. Thus, expression of Impα and Impα–driven nuclear import of Vpr are essential for efficient viral replication in macrophages.

VPR-MEDIATED NUCLEAR IMPORT AS A THERAPEUTIC TARGET

The results described in this review suggest that Vpr-mediated nuclear import is associated with the promotion of viral infection in non-dividing macrophages. Therefore,

strategies to inhibit this Vpr effect could enable the development of anti-HIV-1 therapies with the potential to benefit HIV-1 patients in the future. The simple system of HIV-1 nuclear import *via* the Vpr–Impα interaction has so far been useful in identifying Vpr-specific inhibitors.

HIV-1 infection can be controlled with combinations of anti-retroviral drugs. An approach called highly active anti-retroviral therapy (HAART) uses protease and reverse transcriptase inhibitors that can decrease viremia below the limit of detection and stop disease progression [94, 95]. However, because of problems such as viral escape mutants [96], persistence of viral reservoirs [97-99], poor patient compliance due to complicated drug regimens [100], and toxic side effects [101], the need for new drugs with novel targets has become apparent. Macrophages are the targets of HIV-1 and serve as crucial viral reservoirs [102]. Macrophages are widely distributed in all tissues and organs, where they represent the most commonly HIV–infected cells. In contrast to activated $CD4^+$ T lymphocytes, macrophages are resistant to the cytopathic effects of HIV and survive HIV infection for long periods of time. In addition, HIV-1 in latently infected macrophages cannot be eradicated by HAART [103]. Therefore, new targets for developing novel antiviral agents for HIV-1 replication in macrophages must be identified.

One such target is the nuclear import *via* the Vpr–Impα interaction, as shown in Fig. (**3E**). By targeting this interaction, we successfully identified a novel compound inhibiting both Impα–dependent Vpr nuclear import and HIV-1 replication [104]. Analysis by real-time PCR demonstrated that this drug specifically inhibits the nuclear import of the viral genome, as measured by two-long terminal repeat circle formation, which is marker of nuclear import. In contrast, this drug had only minor effects on Vpr-induced G2/M phase cell cycle arrest and on the nuclear import of karyophilins, which possess a classical basic NLS. Thus, this Vpr-specific inhibitor is a new anti-HIV-1 replication inhibitor that targets the nuclear import of HIV-1 *via* the Vpr–Impα interaction, suggesting that a specific inhibitor of an interaction between a viral protein and a host cellular factor may provide a new therapeutic strategy for blocking HIV-1 replication. This result also suggests that Impα–dependent Vpr nuclear import is a valuable new drug target that can be exploited to develop HIV-1 therapies targeting macrophages, which cannot be eradicated by HAART. Interestingly, two inhibitors (ITI-367 and fz41) that act through IN or MA in the nuclear import of HIV-1 PIC have been reported [105-107]. Thus, nuclear import–blocking drugs may be useful not only against HIV, but also against other viruses that require nuclear import for replication, such as influenza virus, hepatitis B virus, or herpes viruses. The development of such compounds is likely to provide a valuable enrichment of our arsenal of antiviral drugs.

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Novel Nuclear Import of Vpr Promoted by Importin α Is Crucial for Human Immunodeficiency Virus Type 1 Replication in Macrophages[∇]

Yuko Nitahara-Kasahara,[1]† Masakazu Kamata,[1]‡ Takuya Yamamoto,[2] Xianfeng Zhang,[1] Yoichi Miyamoto,[3] Koho Muneta,[1] Sayuki Iijima,[1]§ Yoshihiro Yoneda,[3] Yasuko Tsunetsugu-Yokota,[2] and Yoko Aida[1]*

Retrovirus Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan[1]; Department of Frontier Biosciences, Department of Immunology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan[2]; and Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan[3]

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Monocytes/macrophages are major targets of human immunodeficiency virus type 1 (HIV-1) infection. The viral preintegration complex (PIC) of HIV-1 enters the nuclei of monocyte-derived macrophages, but very little PIC migrates into the nuclei of immature monocytes. Vpr, one of the accessory gene products of HIV-1, is essential for the nuclear import of PIC in these cells, although the role of Vpr in the entry mechanism of PIC remains to be clarified. We have shown previously that Vpr is targeted to the nuclear envelope and then transported into the nucleus by importin α alone, in an importin β-independent manner. Here we demonstrate that the nuclear import of Vpr is strongly promoted by the addition of cytoplasmic extract from macrophages but not of that from monocytes and that the nuclear import activity is lost with immunodepletion of importin α from the cytoplasmic extract. Immunoblot analysis and real-time PCR demonstrate that immature monocytes express importin α at low levels, whereas the expression of three major importin α isoforms markedly increases upon their differentiation into macrophages, indicating that the expression of importin α is required for nuclear import of Vpr. Furthermore, interaction between importin α and the N-terminal α-helical domain of Vpr is indispensable, not only for the nuclear import of Vpr but also for HIV-1 replication in macrophages. This study suggests the possibility that the binding of Vpr to importin α, preceding a novel nuclear import process, is a potential target for therapeutic intervention.

Monocytes/macrophages are major targets of human immunodeficiency virus type 1 (HIV-1) and serve as a viral reservoir (12). Most tissue macrophages are permissive for the entry of macrophage-tropic viruses, and they release small amounts of viral particles in asymptomatic carriers (6). In AIDS patients with opportunistic infections, macrophages occasionally produce large quantities of viral particles (40). Recent studies suggest that HIV-1 in latently infected macrophages in some lymphoreticular tissues cannot be eradicated by highly active antiretroviral therapy and that residual cells may produce viral particles that can spread throughout the body (39). Although the extents of reverse transcription (RT) are similar in monocytes and macrophages after infection with an HIV-based vector, nuclear entry is blocked in monocytes (34). Therefore, it has been considered that active nuclear import of the viral preintegration complex (PIC) of HIV-1 is inefficient in monocytes. However, the precise mechanisms by which the nuclear import of PIC is controlled in monocytes and differentiated macrophages are not fully understood, although this distinction is critical for the design of antiviral strategies.

Nuclear import processes involve the nuclear pore complexes (NPCs) of the nuclear envelope and typically require nuclear localization signals (NLSs). The nuclear import of basic NLS-containing proteins is mediated by specific soluble factors composed of two essential components, importins α and β (13). The central portion of importin α, which contains armadillo repetitive motifs, recognizes the NLS, and its N-terminal basic region, termed the importin β-binding (IBB) domain, binds to importin β (13). The ternary complex docks at the NPC and is translocated into the nucleus. Therefore, importin α acts as an adapter molecule between cargo proteins and importin β, and it is importin β that actually conveys the cargo from the cytoplasm into the nucleus. In addition, several other factors participate in this transport system, including the small GTPase Ran and its binding protein, nuclear transport factor 2 (32). However, there are other pathways that mediate nuclear import; for example, transportin (transport factor of M9-containing cargo) and importin β are competent to transfer some cargo by themselves (41, 46). Moreover, it was recently reported that importin α can migrate into the nucleus in an importin β- and Ran-independent manner (29). In addition, importin α alone can escort Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) into the nucleus without utilizing the classical importin β-dependent transport system (23).

Mammals such as humans and mice possess at least six importin α isoforms (importin α1/Rch1, α3/Qip1, α4/hSRP1γ, α5/NPI1, α6, and α7 [8, 21, 22, 38, 44]). These isoforms can be divided into the following three major subfamilies according to their amino acid similarities: α1, α3 and α4, and α5 to α7 (22).

* Corresponding author. Mailing address: Retrovirus Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Phone: 81 48 462 4408. Fax: 81 48 462 4399. E-mail: aida@riken.jp.

† Present address: National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.

‡ Present address: University of California, Los Angeles, CA 90095.

§ Present address: National Institute of Biomedical Innovation, Hachimandai 1-1, Tsukuba, Ibaraki 305-0843, Japan.

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Proteins in the three groups share about 50% overall amino acid identity. Many studies have shown that importin α isoforms differ in efficiency with respect to classical substrate-specific import (31, 45) and show unique expression patterns in various tissues. This suggests that importin α contributes primarily to tissue-specific nuclear transport. However, the expression patterns of importin α isoforms in human peripheral blood mononuclear cells (PBMC) are unknown.

The ability of HIV-1 to replicate in nondividing cells, such as macrophages, depends on the active nuclear import of the viral PIC (4). The HIV-1 PIC contains viral proteins such as reverse transcriptase, integrase (IN), nucleocapsid (NC), Vpr, and matrix (MA; p17) in addition to viral nucleic acids (5). MA, Vpr, and IN have all been implicated in the nuclear import of PIC, although their precise roles are controversial. Both MA and IN have functional NLSs that resemble the canonical NLS of the simian virus 40 (SV40) T antigen, and both utilize the classical nuclear import pathway that includes interaction with importins α and β (11). In contrast, despite the lack of an identifiable canonical NLS, Vpr displays karyophilic properties and is rapidly targeted to host cell nuclei after infection (26). Furthermore, it has been reported that the nuclear import of Vpr is mediated by an as-yet-unidentified pathway that is distinct from the classical NLS- and M9-dependent pathways (16). In this context, we have previously shown that Vpr traverses the NPC in an importin α-dependent manner (19). Vpr has also been implicated in the nuclear import of proviral DNA in macrophages (7, 11, 14), presumably by promoting interactions with the cellular machinery that regulates nucleocytoplasmic shuttling (10, 14, 25, 42, 43, 48). In addition to nuclear transport, Vpr functions in many processes, including the induction of cell cycle arrest at the G_2 phase (17), the regulation of apoptosis (2, 3, 35–37), and splicing (24). The numerous biological activities of Vpr appear to be related to its interactions with a variety of cellular partners. Indeed, it has been suggested that importin α binds to Vpr (1, 42, 43, 48) to promote its passage through the NPC (19). We have previously shown that the region between residues 17 and 74 of Vpr, designated N17C74, is a bona fide NLS. In addition, Vpr seems to be targeted first to the NPC via interaction with the αH3 region, located between residues 46 and 74, and then enters the nucleus in a process that involves the αH1 region, located between residues 17 and 34 (19). However, it remains to be clarified whether Vpr is transported into the nucleus by importin α alone, without the need for other soluble factors, such as importin β.

In this investigation, we have studied the detailed mechanism of Vpr nuclear import and its correlation with HIV-1 infectivity in primary monocytes and macrophages. We demonstrate the following. (i) In digitonin-permeabilized cells, Vpr alone is targeted to the perinuclear region and then transported into the nucleus by importin α in an importin β-independent manner. (ii) The three major isoforms of importin α support the apparent nuclear import of Vpr. (iii) Primary monocytes exhibit a marked increase in the expression of importin α isoforms upon induction of differentiation to macrophages. (iv) The expression of importin α is essential for the nuclear import of Vpr in macrophages. (v) The interaction between importin α and Vpr is indispensable, not only for the importin α-mediated nuclear import of Vpr but also for the replication of HIV-1 in primary macrophages.

MATERIALS AND METHODS

Cell culture, cytoplasmic extract preparation, and RNA extraction. Human cervical HeLa cells and African green monkey COS cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Human PBMC were isolated on a Ficoll (Lymphosepal; IBL) gradient from a healthy HIV-1-seronegative donor. Monocytes were selected from the PBMC by a magnetic cell separation system, using microbeads coated with a CD14-specific monoclonal antibody (MAb), according to the manufacturer's instructions (MACS system; Miltenyi Biotech). Monocytes were plated at the desired density in 5-mm-diameter poly-L-lysine-coated glass-bottomed microwell dishes (Matek Corp.) and grown in RPMI medium (Invitrogen) containing 10% heat-inactivated fetal calf serum, 5% human serum, and 20 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech) for 1 week, until they spontaneously differentiated into mature macrophages (34).

Cells were lysed in cold, hypotonic buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.3 M sucrose, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cytoplasmic extracts were clarified by centrifugation at 8,000 × *g* for 30 s, and the supernatants were subjected to Western blotting analysis and an in vitro nuclear transport assay.

Total cellular RNAs were isolated from monocytes and differentiated primary macrophages by using TRIzol reagent (Invitrogen).

Plasmid constructions. Construction of glutathione *S*-transferase (GST)- and green fluorescent protein (GFP)-tagged mutant forms of Vpr, named N17C74-GFP and GST-αLA/N17C74-GFP, was done as previously described (15, 19). For construction of GST-αH1-GFP and GST-αLA/αH1-GFP, fragments encoding the sequences were prepared from pGFP-FαH1 and pGFP-FαLA/αH1, respectively, by digestion with EcoRV and NotI (18). Each fragment was subcloned into BamHI/NotI-digested pGEX-6P3 (GE Healthcare). The BamHI site was blunted with KOD DNA polymerase (Toyobo) for ligation with an EcoRV site. GST-tagged human importin α1, α3, and α5 isoforms were constructed as follows. Insert fragments were isolated from pGEX-2T/importin α1, pGEX-2T/importin α5, and pGEX-2T/importin α3 and subcloned into pGEX-6P3 (30). This vector includes the GST coding region and a Flag tag, at the N and C termini of the multicloning site, respectively. For the importin α1 deletion mutant that lacked the IBB domain, the ΔIBB importin α fragment was amplified with the primers 5′-TATGGATCCAGCTCCTTTCCTGAT-3′ and 5′-GGCCTCGAGGTAAAAGTTAAAGGTCCCAGG-3′, using pGEX-6P3/importin α1 as the template. GST- and hemagglutinin-tagged human importin β was cloned into pGEX-2T at the BamHI and KpnI sites. This fragment was then subcloned into pGEX-6P3 at the BamHI and XhoI sites. The GST-tagged SV40 NLS-GFP construct was made as previously described (19). An infectious molecular clone, HIV-1 pNF462, a clone that encoded a Vpr-negative ATG mutant (ΔVpr), and a clone that encoded a substitution mutant of Vpr designated αLA were constructed as previously described (15).

Expression and purification of recombinant proteins. GST- and GFP-tagged mutant forms of Vpr and GST-tagged importin α1, α3, α5, and β were expressed in *Escherichia coli* strain NovaBlue (Novagen) or BL21 CodonPlus (DE3)-RIL (Stratagene) and purified as described elsewhere (15, 19). GST-tagged SV40 NLS-GFP (18) and Ran/TC4 (28) were also expressed in *E. coli* and purified as described previously (15, 19).

Western blotting. Cell lysates were examined by immunoblotting with a MAb against importin α1 (BD Biosciences), a MAb against importin α3 (MBL), a MAb against importin α5/7 (MBL), a MAb against importin β (BD Biosciences), a MAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; BD Biosciences), an anti-HIV-1 Vpr rabbit serum (NIH AIDS Research and Reference Reagent Program), or a MAb against HIV-1 Gag (p24) (15), followed by horseradish peroxidase-linked sheep antibodies against mouse or rabbit immunoglobulin G (IgG; GE Healthcare), as previously described (37).

Quantitative real-time PCR. Approximately 600 ng total RNA was used for the RT reaction, which was performed with the Superscript preamplification system (Invitrogen). The RT product was used for real-time quantitative PCR amplification of three importin α isoforms in a LightCycler system (Roche) in the presence of LightCycler FastStart DNA SYBR green I master mix (Roche), using importin α1, α3, and α5 isoform-specific primers as follows: forward α1, 5′-GCATAATAGAACCGTTGATG-3′; reverse α1, 5′-AGGAGCCCCATCCTGAAC-3′; forward α3, 5′-AGTGGCTTACCTTATCCAAC-3′, reverse α3, 5′-TGTTGGTACATTGGCAGATG-3′; forward α5, 5′-GTGATCTCCTCACGGTCATG-3′; and reverse α5, 5′-CATAGGAGCCTCACACTG-3′. Hypoxanthine

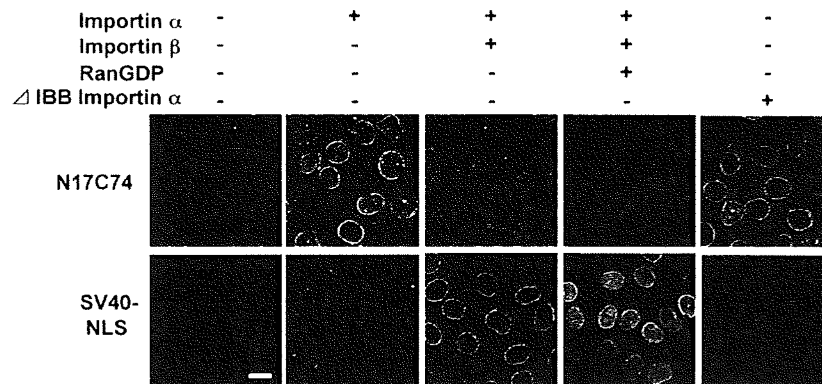

FIG. 1. Importin α mediates the nuclear import of Vpr without importin β. HeLa cells were permeabilized by treatment with 35 μg/ml digitonin. The cells were incubated with 1 μM GST- and GFP-tagged N17C74 or SV40 NLS in the presence (+) or absence (−) of soluble factors for 20 min at 30°C. After fixation, cells were analyzed by confocal laser scanning microscopy (Radiance 2100; Bio-Rad). Soluble factors were included at the following concentrations: importin α1, 1 μM; importin β, 1 μM; RanGDP, 2 μM; and ΔIBB importin α1, 1 μM. Bar = 20 μm.

phosphoribosyltransferase (HPRT) cDNA was amplified as a normalization control, using the primers forward HPRT (5′-GCCCTGGCGTCGTGATTAGT-3′) and reverse HPRT (5′-GCTCTACTAAGCAGATGGCC-3′).

Microinjection and imaging analysis. GST and GFP fusion proteins (1 mg/ml) were injected into the cytoplasm of primary macrophages grown on a 35-mm glass-bottomed dish, using an InjectMan N12 microinjector (Eppendorf). Images were captured with a 40× long-distance objective lens on an Olympus IX70 inverted microscope equipped with a Yokogawa CSU10 confocal laser scanning system controlled by Metamorph software (Universal Imaging).

siRNA transfection. Small interfering RNAs (siRNAs) corresponding to importins α1, α3, α5, and β were designed with BLOCK-iT RNAi Designer (Invitrogen) and obtained. The siRNA sequences targeting importins α1, α3, α5, and β were CCAAGCUACUCAAGCUGCCAGGAAA for α1, CAGUGAUC GAAAUCCACCAAUUGAU for α3, CCGGAAUGCAGUAUGGGCUUU GUCU for α5, and CAGUCUGGCUGAAGCUGCUUAUGAA and CACAG CACUGCAGUCUGGAUUCCUU for β. HeLa cells were seeded 1 day before transfection with siRNAs. On day 0, transfection of importin α1-, α3-, α5-, and β-specific siRNAs, an HPRT-specific siRNA (HPRT-S1) as a positive control, or a nonspecific siRNA as a negative control was carried out with Lipofectamine RNAiMAX (Invitrogen) following the company protocol. After 2 days, second transfections were performed. After another 2 days, cells were harvested, and cytoplasmic extracts were prepared. Transfection efficiencies for siRNAs were determined by using Western blotting with MAbs against importins α1, α3, α5/7, and β.

HIV-1 infection of macrophages. HIV-1 was introduced into COS cells by electroporation of macrophage-tropic pNF462 viruses encoding wild-type Vpr, a mutant form (αLA), and a deficient form. Viral supernatants were harvested 72 h after transfection. Virus stocks were titrated by measuring the amount of p24 antigen in the culture supernatants, using an enzyme-linked immunosorbent assay (ELISA). Differentiated primary macrophages seeded onto a 48-well tissue culture plate (2×10^5/well) were exposed to virus containing 5 ng of p24 antigen for 2 h at 37°C. They were then washed three times, and the infected cells were maintained in RPMI 1640 containing 10% fetal calf serum, 5% human serum, and M-CSF (20 ng/ml). Culture supernatants were harvested at 3- or 4-day intervals, and viral production was monitored by the sequential quantification of p24 antigen in cell-free supernatants with an HIV-1 $p24^{gag}$ ELISA kit (ZeptoMetrix Corp.).

Other assays. In vitro nuclear transport and in vitro pull-down assays were performed as previously described (15, 19).

RESULTS

Nuclear import of Vpr is promoted by importin α without a requirement for importin β. We previously reported that Vpr is localized at the nuclear envelope and that it then traverses the NPC in an importin α-dependent manner (19). To determine whether the human importin α-driven nuclear import of Vpr requires importin β, we reconstituted the candidate factors required for Vpr nuclear transport from digitonin-permeabilized, semi-intact HeLa cells. For this experiment, we used the region between residues 17 and 74 (N17C74) of Vpr because this is a functionally transportable region (18, 19). The chimeric protein comprised of N17C74 fused at the N terminus to GST and at the C terminus to GFP (~63 kDa) was larger than the limitation for passive diffusion into the nucleus. As shown in Fig. 1, N17C74 localized predominantly to the perinuclear region in the absence of soluble factors. Interestingly, importin α1 alone had the highest activity for the nuclear import of N17C74, whereas the addition of importin β decreased the efficiency of N17C74 import, in the presence or absence of RanGDP (Fig. 1, upper panels). These results contrast with the classical nuclear import of SV40 NLS, which requires importin α1, importin β, and RanGDP (Fig. 1, lower panels), but are in good agreement with our recent report (19). Next, to exclude the possibility that residual endogenous importin β in the permeabilized cells contributed to the nuclear migration of Vpr, an importin α1 mutant (ΔIBB) lacking the IBB domain and unable to bind to importin β was used instead of full-length importin α1. ΔIBB importin α1 alone enhanced the nuclear import of N17C74, indicating that Vpr can enter the nucleus in an importin β-independent manner.

These results support the notion that Vpr is targeted to the perinuclear region and then transported into the nucleus by importin α1 alone, without importin β. Thus, the mechanism of nuclear entry of Vpr is quite different from that mediated by the classical transport system.

Nuclear import of Vpr is promoted by all three major isoforms of importin α. In human cells, at least six importin α isoforms have been identified, and they are believed to differ in efficiency with respect to classical substrate-specific import (31, 45). To determine whether the other two major importin α isoforms, α3 and α5, promote the nuclear import of Vpr similarly to α1, we performed an in vitro transport assay using recombinant human α1, α3, and α5. As shown in Fig. 2A, N17C74 was imported into the nucleus by either importin α3 or α5 as well as by α1, suggesting that Vpr is able to utilize all three major importin α isoforms for nuclear entry. The classical nuclear import of SV40 NLS, the positive control for the in

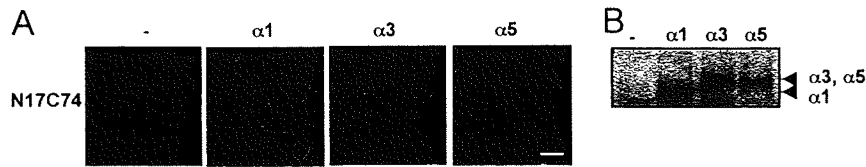

FIG. 2. Importin α1, α3, and α5 isoforms interact with Vpr and mediate the nuclear import of Vpr. (A) Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 and 1 μM of each recombinant importin α isoform. After fixation, cells were analyzed by confocal laser scanning microscopy. Bar = 20 μm. (B) Glutathione-Sepharose beads coupled with 100 pmol GST- and GFP-tagged N17C74 were incubated with 100 pmol recombinant GFP-tagged importin α1, α3, or α5. After incubation for 1 h at 4°C, the bound fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie staining. The positions of the three importin α isoforms are indicated.

vitro transport assay, occurred in the presence of any of the three importin α isoforms in combination with importin β and RanGDP (data not shown).

To further examine whether N17C74 interacts directly with the three importin α isoforms, recombinant importin α1, α3, and α5 were incubated with GST- and GFP-tagged N17C74 that was immobilized on glutathione-Sepharose (Fig. 2B). Although the three isoforms share only approximately 50% overall amino acid sequence similarity, the tagged N17C74 protein was able to interact with all of them. These results suggest that the three importin α isoforms can directly interact with Vpr and support its nuclear entry.

Importin α in differentiated macrophages promotes the nuclear import of Vpr. Vpr has been implicated as playing an important role in the nuclear import of proviral DNA in monocyte-derived macrophages (7, 11, 14). Neil et al. reported that although the HIV-1 PIC entered the nuclei of primary monocyte-derived macrophages, active nuclear import of PIC was

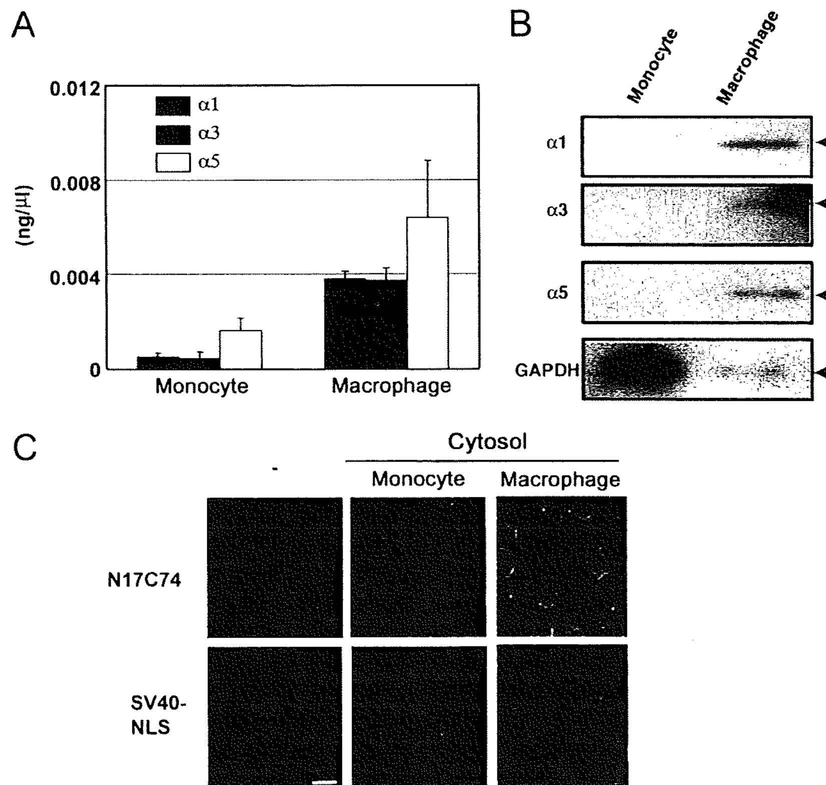

FIG. 3. Importin α in cytoplasmic extracts from primary macrophages promotes the nuclear import of Vpr. Monocytes were isolated from human PBMC. Macrophages were differentiated from monocytes by M-CSF. (A) Detection of importin α isoform mRNAs by real-time quantitative PCR. Total RNAs were extracted from monocytes and differentiated primary macrophages, and quantitative RT-PCR analysis was performed on a LightCycler system, using specific primers for each importin α isoform. Bars represent the mean values and standard errors for three experiments. (B) Detection by Western blotting of importin α proteins. Lysates containing 100 μg protein from monocytes and differentiated primary macrophages were subjected to Western blotting with MAbs against importins α1, α3, and α5 and against GAPDH as a control. The positions of the three importin α isoforms and GAPDH are indicated. (C) In vitro nuclear transport assay. Cytoplasmic extracts were prepared from monocytes and differentiated primary macrophages. Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 or GST- and GFP-tagged SV40 NLS and extracts containing 100 μg protein. After fixation, cells were analyzed by confocal laser scanning microscopy. Bar = 20 μm.

inefficient in monocytes (34). These results suggested that the efficiency of HIV-1 PIC nuclear import in monocytes and macrophages might correlate with that of Vpr. However, it was unclear whether importin α was expressed in monocytes and macrophages, interacted with Vpr, and contributed to HIV-1 replication. Therefore, we first investigated the mRNA expression level of each importin α isoform in primary monocytes and differentiated macrophages. Primary monocytes were prepared from PBMC of healthy donors and allowed to differentiate into macrophages in vitro in the presence of M-CSF. Total cellular RNA was isolated from primary monocytes and macrophages, and real-time quantitative RT-PCR was conducted using specific primers for each isoform (Fig. 3A). Differentiated macrophages showed higher levels of expression of all three isoforms than did monocytes. Differences in the protein levels of each importin α isoform between monocytes and macrophages were confirmed by Western blotting with MAbs against importin α1, α3, and α5 (Fig. 3B). These results were consistent with the observed levels of mRNA expression. These data clearly show that the expression of the three major isoforms of importin α is low in primary monocytes but is markedly increased by inducing differentiation into macrophages.

Next, we performed an in vitro nuclear transport assay using cytoplasmic extracts from monocytes and differentiated macrophages (Fig. 3C). As expected from the above results, N17C74 was efficiently transported into the nucleus when a cytoplasmic extract from differentiated macrophages was added, whereas the nuclear import of N17C74 was inefficient when a monocyte extract was used. Under these conditions, the classical nuclear import of SV40 NLS was also inefficient with monocyte extracts and efficient with macrophage extracts. These results suggest that the efficiency of nuclear transport of Vpr and SV40 NLS depends strongly on the expression level of importin α in monocytes and macrophages, which are important targets for HIV-1 infection.

Importin α depletion prevents the nuclear import of Vpr in macrophages. To further investigate the requirement for importin α in the nuclear import of Vpr in primary macrophages, cytoplasmic extracts were depleted, using a MAb against importin α1 and protein A-Sepharose, prior to the in vitro nuclear transport assay. Western blotting analysis of depleted lysates unexpectedly demonstrated that most importin α, including importins α3 and α5 as well as importin α1, was successfully removed, whereas importin β remained in the extracts (Fig. 4A). The addition of these extracts drastically decreased the nuclear import of N17C74 compared to that observed with cytoplasmic extracts incubated with preimmune normal mouse IgG (Fig. 4B). Similar results were obtained with SV40 NLS as a control for classical import. In addition, we successfully knocked down the three importin α isoforms α1, α3, and α5 in HeLa cells by isoform-specific siRNA transfection (Fig. 5A). The nuclear import of N17C74, which was enhanced by a cytoplasmic extract of HeLa cells, was greatly decreased by a cytoplasmic extract from importin α-specific siRNA-transfected cells but not by that from importin β-specific, HPRT-specific, or negative control siRNA-transfected cells (Fig. 5B).

These results suggest that endogenously expressed importin α is essential for the efficient nuclear import of Vpr.

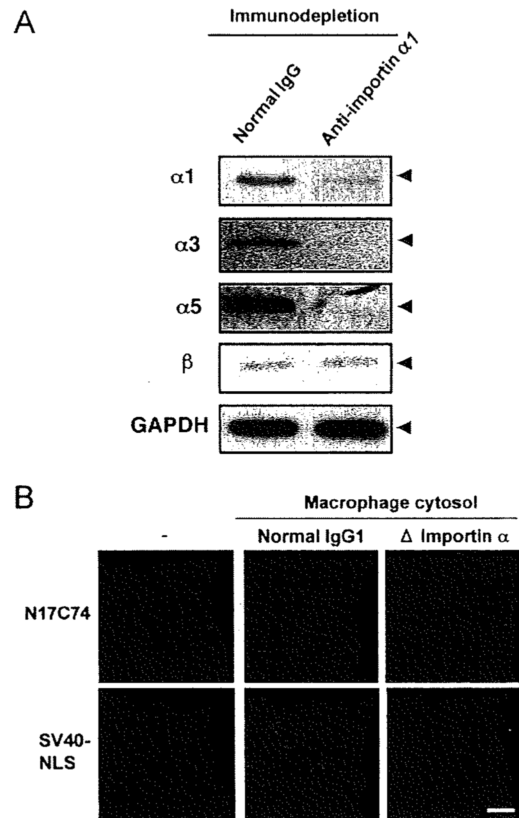

FIG. 4. Importin α depletion prevents the nuclear import of Vpr in macrophages. (A) Lysates of differentiated primary macrophages were treated with a MAb against importin α1 or with preimmune normal mouse IgG, followed by treatment with protein A-Sepharose. The cytoplasmic extracts were clarified by centrifugation, and equivalent amounts of the immunodepleted extracts were subjected to Western blotting with MAbs against importins α1, α3, and α5, importin β, or GAPDH. The positions of the three importin α isoforms, importin β, and GAPDH are indicated. (B) In vitro nuclear transport assay. Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 or SV40 NLS and 100 μg of control or immunodepleted cytoplasmic extract. After fixation, cells were analyzed by confocal laser scanning microscopy. Bar = 20 μm.

Interaction of importin α with the αH1 domain of Vpr is essential for its nuclear import in macrophages. To understand the mechanism of the importin α-driven nuclear import of Vpr in detail, we examined the effects of mutations on the nuclear import of Vpr. Vpr consists of three α-helical domains (αH1, αH2, and αH3) (Fig. 6A). As we showed previously, N17C74 interacts directly with importin α1 through the αH1 and αH3 domains, and the interaction via αH1 is essential for nuclear entry (19). Therefore, in this study, we focused on the αH1 domain interaction. Three mutants were used in this study, including αLA/N17C74, in which the Leu residues at positions 20, 22, 23, and 26 within αH1 of Vpr were replaced by Ala residues; the αH1 domain alone; and its mutant, αLA/αH1 (Fig. 6A). In pull-down assays, we found that the αH1 chimeric protein interacted strongly with endogenous importin α in the cytoplasmic extract of HeLa cells, whereas the αLA/αH1 protein interacted very poorly. A similar result was obtained with recombinant importin α (Fig. 6B). These results

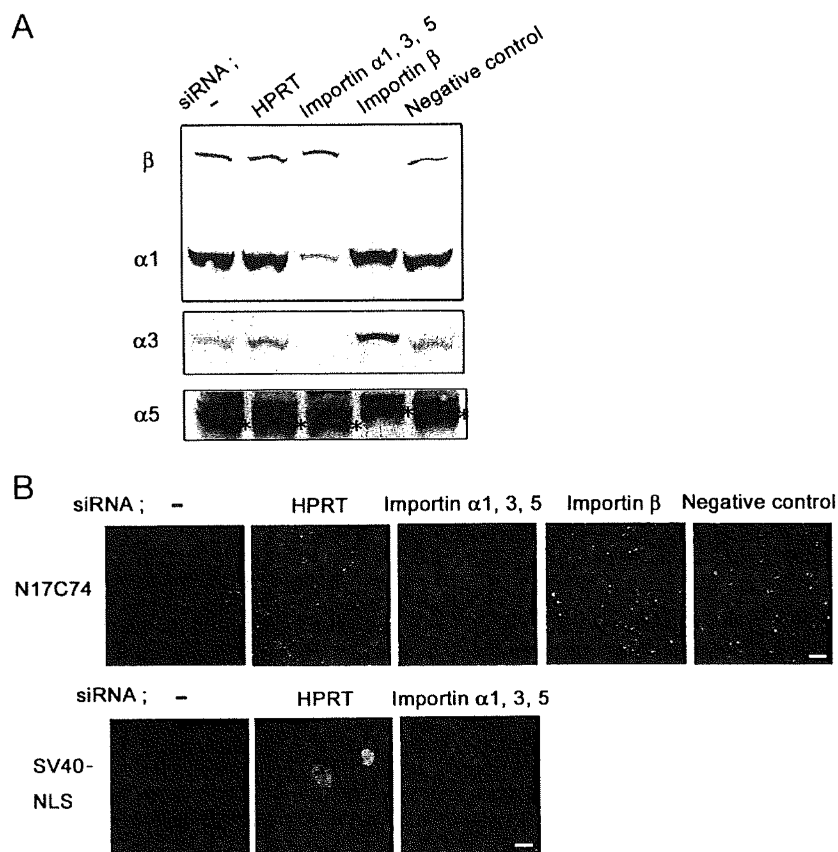

FIG. 5. siRNA-induced down regulation of importin α isoforms prevents the nuclear import of Vpr. (A) Duplicate siRNA transfections corresponding to importins α1, α3, α5, and β, HPRT-specific siRNA transfection as a positive control, and nonspecific siRNA transfection as a negative control were carried out with HeLa cells. Cytoplasmic extracts were prepared from HeLa cells transfected with siRNA. The transfection efficiencies of siRNAs were determined by Western blotting using importin α1-, α3-, α5/7-, and β-specific antibodies. The positions of importins α1, α3, α5 (*), and β are indicated. (B) In vitro nuclear import assay. Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 or GST- and GFP-tagged SV40 NLS and 100 μg proteins from a cytoplasmic extract prepared from HeLa cells transfected with siRNA. After fixation, cells were analyzed by confocal laser scanning microscopy. Bar = 20 μm.

indicate that the Leu residues at positions 20, 22, 23, and 26 in the αH1 region of Vpr are crucial for binding to importin α.

To further demonstrate the importance of this region for the nuclear transport of Vpr, we carried out an in vitro nuclear transport assay with the αLA/N17C74 chimeric protein, using digitonin-permeabilized HeLa cells. As shown in Fig. 6C, the αLA/N17C74 mutant had completely lost the ability to migrate into the nucleus in the presence of a cytoplasmic extract prepared from primary macrophages, even in the presence of recombinant importin α. The localization of N17C74 and of the αLA/N17C74 mutant protein was determined after cytoplasmic microinjection into primary macrophages. N17C74 clearly localized in the nucleus, whereas the mutant did not, suggesting that the mutant had lost nuclear import activity (Fig. 6D). These findings clearly indicate that the ability of Vpr to interact with importin α is indispensable for its nuclear import in macrophages.

Interaction of importin α through the αH1 domain of Vpr is crucial for the efficient replication of HIV-1 in macrophages. Finally, it was important to determine whether the nuclear entry of Vpr mediated by its interaction with importin α is crucial for macrophage-tropic HIV-1 replication in primary macrophages. We compared the replication in primary macrophages of HIV-1 carrying wild-type Vpr, a Vpr ATG mutant (ΔVpr), and a mutant form (αLA) that cannot interact with importin α and thus is defective in nuclear transport. The αLA mutant retained the ability to induce G_2 arrest and apoptosis (data not shown). To examine whether the αLA mutant virus synthesized mutant Vpr protein, HeLa cells were transfected with pNF462 proviral DNA encoding wild-type Vpr or the Vpr mutant form (αLA), and the cell lysates were analyzed by Western blotting using anti-Vpr antibody and anti-Gag antibody. The wild-type and αLA mutant transfectants had similar amounts of Vpr and $p24^{Gag}$ (Fig. 7A). Typical kinetics for the replication of these wild-type and mutant viruses in the primary macrophages of two donors are shown in Fig. 7B. The virus carrying wild-type Vpr replicated well in both donors, with viral inputs of 5 ng p24 antigen, and replication reached a peak 14 to 17 days after infection. In contrast, ΔVpr HIV-1 displayed a markedly decreased level of replication compared to the wild-type virus, suggesting the importance of Vpr in HIV-1 replication in primary macrophages. Interestingly, replication of the αLA mutant virus was also reduced to a level equivalent to that of the ΔVpr virus. Similar results were ob-

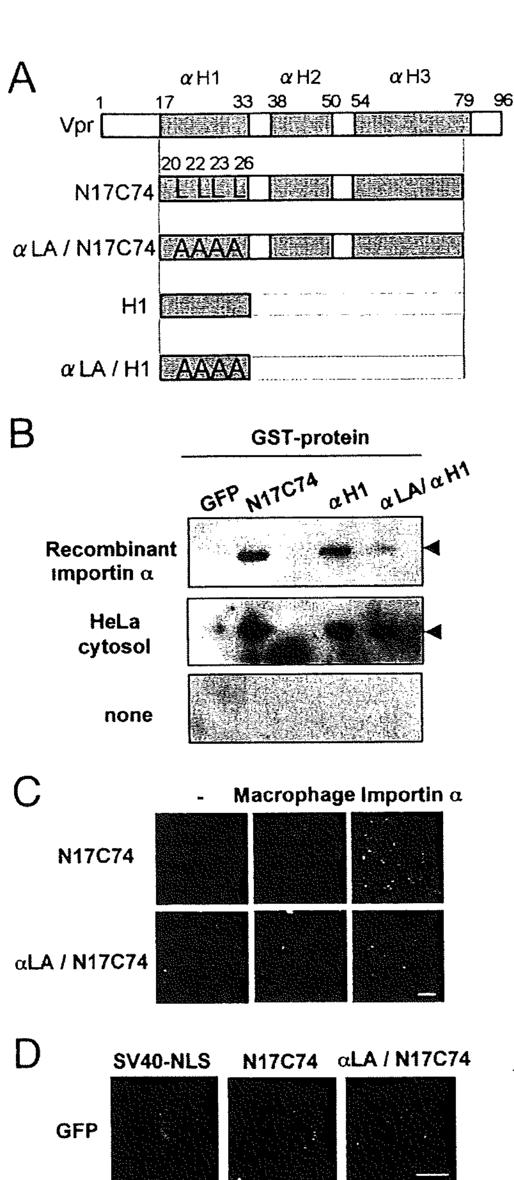

FIG. 6. Vpr mutants that cannot bind importin α are not imported into the nucleus. (A) Construction of plasmids encoding GST- and GFP-tagged mutant forms of Vpr. The three α-helical domains (αH1, αH2, and αH3) are represented by shaded boxes. The Leu residues at positions 20, 22, 23, and 26 in the αH1 domain were replaced by Ala. (B) Glutathione-Sepharose beads were coupled with the recombinant proteins, namely, GST-tagged GFP, GST-N17C74-GFP, GST-αH1-GFP, and GST-αLA/αH1-GFP, and incubated with 100 pmol recombinant importin α (top), 100 μg cytosol of HeLa cells (middle), or nothing as a control (bottom). The proteins recovered from the beads were subjected to Western blotting with a MAb against importin α1. The position of importin α is indicated. (C) Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 or αLA/N17C74 in the presence of 100 μg cytoplasmic extract prepared from primary macrophages or 1 μM recombinant importin α. After fixation, cells were analyzed by confocal laser scanning microscopy. Bar = 20 μm. (D) GST- and GFP-tagged N17C74, αLA/N17C74 or SV40 NLS was injected into the cytoplasm of differentiated primary macrophages grown on a glass-bottomed dish. After 15 min, the transport reactions were captured by confocal laser scanning microscopy. Bar = 20 μm.

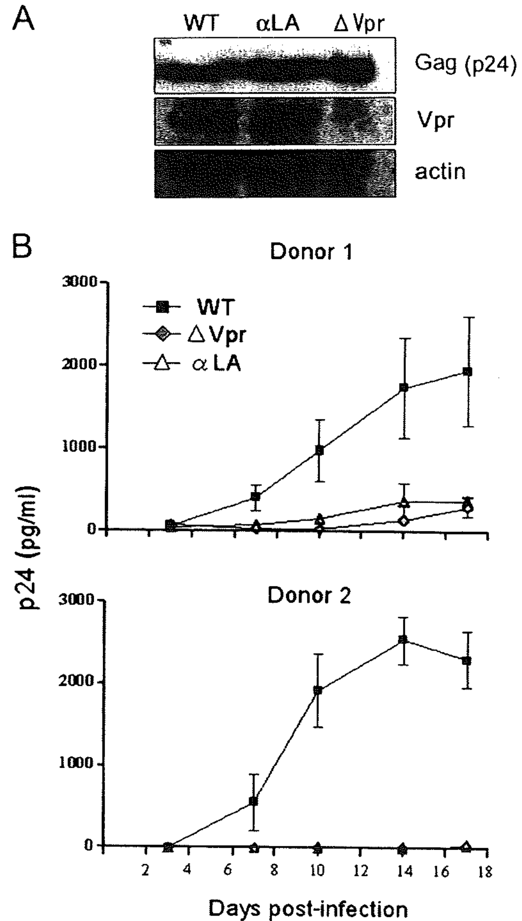

FIG. 7. Interaction between Vpr and importin α is crucial for the efficient replication of macrophage-tropic HIV-1 in macrophages. (A) Cell lysates derived from HeLa cells transfected with pNF462 proviral DNA encoding wild-type Vpr (WT), the Vpr mutant form (αLA), or a deficient form of Vpr (ΔVpr) were analyzed by Western blotting with anti-Vpr and anti-Gag antibodies. (B) Differentiated primary macrophages from two independent donors were infected with macrophage-tropic viruses (5 ng/well) that encoded wild-type Vpr (■), the mutant αLA form (Δ), or a deficient form of Vpr (◆), and the kinetics of virus production were analyzed. Cells were maintained for 3 weeks, and the levels of virus production in culture supernatants were measured by p24 antigen ELISA. All samples were tested in triplicate, and the data presented are the mean levels of p24 antigen. The bars indicate the standard errors of the measurements.

tained with macrophages isolated from four additional donors (data not shown).

Our results strongly support the notions that the binding of Vpr to importin α is essential for the nuclear import of Vpr and that this nuclear import is crucial for viral replication in macrophages. In addition, our results indicate that expression of importin α is essential for efficient viral replication in macrophages.

DISCUSSION

Novel nuclear import mechanism for Vpr promoted by importin α. We previously showed that Vpr traverses the NPC in an importin α-dependent manner (19). Our present study

clearly demonstrates that importin α promotes nuclear import of Vpr in digitonin-permeabilized cells, without the need for importin β or other soluble proteins. The importin α derivative ΔIBB, which cannot bind to importin β, also promotes nuclear import of Vpr. In addition, depletion of three importin α isoforms from HeLa cells by use of siRNAs markedly decreased the nuclear import of Vpr in an in vitro nuclear transport assay using cytoplasmic extracts but not in importin β-depleted extracts. These results strongly suggest that Vpr is transported into the nucleus by importin α alone, without utilizing the classical importin β-dependent transport pathway. To our knowledge, Vpr is the first retroviral protein that has been shown to use a nuclear import mechanism involving importin α alone, without any other soluble factors. Moreover, as previously reported (19), Vpr directly localizes to the perinuclear region, without a requirement for any soluble factors, before it is transported into the nucleus by importin α. This perinuclear localization distinguishes the nuclear import of Vpr from that of other NLS-bearing proteins, suggesting that the karyophilic properties of Vpr rely on a novel mechanism.

Our present and previous results enable us to characterize the nuclear import of Vpr as follows. (i) Vpr is targeted to the perinuclear region in the absence of soluble factors, as shown by in vitro transport assays (Fig. 1) (19). (ii) The transport of Vpr is mediated by importin α alone, without the intervention of importin β, transportin, RanGDP, or NTF2 (Fig. 1) (19). Moreover, the ΔIBB derivative of importin α can also promote nuclear import of Vpr, confirming the transport of Vpr by importin α without the aid of importin β (Fig. 1). (iii) Vpr interacts with importin α through two α-helical domains, αH1 and αH3, which play distinct roles in nuclear import (18, 19). (iv) Vpr interacts with the C-terminal region of importin α, and this interaction is required for nuclear entry (19). (v) The nuclear import of Vpr is promoted by the three major isoforms of importin α (Fig. 2). It was recently reported that importin α alone can carry CaMKIV into the nucleus without utilizing importin β (23). Similar to the case for Vpr, the nuclear transport of CaMKIV requires the C-terminal region of importin α and is promoted by all three major isoforms of importin α. We have found that Vpr and CaMKIV share a highly conserved amino acid region that is rich in Leu and Gln residues. In our study, the αLA mutant, in which all Leu residues are replaced with Ala in the αH1 domain, is incapable of nuclear import due to its reduced interaction with importin α. In addition, this mutant appears to retain not only its G_2 arrest and apoptosis-promoting activities, at half the levels for wild-type Vpr (data not shown), but also the same secondary profile as wild-type Vpr, as calculated by a secondary structure prediction program (27). Therefore, this Leu-rich region may be important for the atypical nuclear import promoted by importin α alone. Additional analysis will be required to completely elucidate this novel import mechanism.

We have previously demonstrated that the perinuclear localization of Vpr is necessary for its nuclear transport (19). In the present study, Vpr appeared to be targeted to the perinuclear region, without the assistance of soluble factors, prior to entering the nucleus in an importin α-dependent manner, which is notably distinct from the mechanism of CaMKIV nuclear import mediated by importin α. Earlier studies have shown that Vpr binds to several nucleoporins, including human p54 and p58 Nups, rodent POM121, *Saccharomyces cerevisiae* NUP1P, and human CG1 (10, 25, 42, 48). It has been suggested that Vpr itself may directly dock on the NPC before entry into the nucleus and that this is the focal point of the new nuclear import mechanism. Therefore, further study is required to define the role of Vpr docking to the nuclear envelope for nuclear entry.

It is known that various importin α isoforms can recognize the same target proteins, although each isoform differs in its efficiency for importing NLS-bearing proteins. Our study shows that all three major importin α isoforms, i.e., α1, α3, and α5, can promote nuclear import of Vpr and that the efficiency of import depends on the expression level of importin α in monocytes and macrophages. This result implies that Vpr enters the nucleus by a mechanism that is common to importin α isoforms. Although the N-terminal IBB domain and the C-terminal domain of importin α isoforms exhibit approximately 50 to 85% amino acid identity, all isoforms interact with importin β and the cellular apoptosis susceptibility gene product (CAS) via their IBB and C-terminal domains (22). In contrast, Vpr can bind these two domains of importin α, and binding with the C-terminal region is essential for import (19). Likewise, the nuclear import of CaMKIV requires the C terminus of importin α. The C-terminal region of importin α presumably contributes to the nonclassical import that utilizes importin α alone.

Importin α is essential not only for the nuclear import of Vpr but also for viral replication in macrophages. The levels of RT are similar in monocytes and differentiated macrophages after infection with the HIV-based vector, whereas there are no detectable two-long-terminal-repeat vector circles in monocytes, suggesting that the nuclear entry of this vector is blocked in monocytes (34). Based on the present study, we propose a possible mechanism to explain the inhibition of PIC nuclear entry in monocytes. Firstly, we have shown that the three major importin α isoforms are abundantly expressed in differentiated macrophages but poorly expressed in monocytes. Secondly, in vitro transport assays and in vivo microinjection experiments have demonstrated that the nuclear import of Vpr correlates well with the expression level of importin α. Thirdly, the requirement for importin α for Vpr nuclear import in macrophages has been confirmed by the observation that depletion of importin α from cytoplasmic extracts of macrophages prevents import. In addition, in vitro nuclear transport assays demonstrated that siRNA depletion of importin α from cytoplasmic extracts of HeLa cells markedly decreases Vpr nuclear import. Thus, we can conclude that importin α is required for the nuclear import of Vpr. Finally, experimental infection with a virus encoding a Vpr mutant protein that cannot bind importin α showed that the expression of importin α is essential for viral replication in macrophages. Although the low level of importin α in monocytes may also be the cause of the inefficient nuclear import of MA and IN, which utilize the classical importin α/β-dependent nuclear import pathway (11), the reduced replication of *vpr*-deficient HIV-1 indicates the importance of Vpr in PIC nuclear import in primary macrophages (Fig. 7) (7, 14). Taken together, these results show that there is a good possibility that Vpr nuclear import is inefficient in monocytes, which do not express importin α, and that therefore the PIC cannot enter the nuclei of these cells.

Importin α isoforms have been shown to differ in their cell- and tissue-specific expression patterns and to depend on the state of cellular metabolism and differentiation (20, 30, 47). Indeed, it has been reported that importins α1 and α5 are inducible and differentially expressed in the human Jurkat and Raji lymphocyte lines (33) and, in addition, that importin α isoforms are expressed in the human leukemia HL60 cell line during proliferation and differentiation into macrophages or neutrophils (21). However, the expression of importin α isoforms has not been investigated previously in human primary PBMC. In the present study, we demonstrated that importin α isoforms α1, α3, and α5 are more abundantly expressed in primary differentiated macrophages prepared from healthy donors than in undifferentiated monocytes, indicating that primary monocytes undergo a marked increase in the expression of these three isoforms upon differentiation. In addition, we have found that all three major importin α isoforms are more strongly expressed in activated $CD4^+$ T cells than in resting $CD4^+$ T cells (unpublished data). This result may provide significant information about the cell-specific expression of importin α. Further study is required to clarify whether or not other importin α isoforms, such as α4, α6, and α7, expressed in monocytes, macrophages, resting $CD4^+$ T cells, and activated $CD4^+$ T cells are targets of HIV-1 infection.

Importin 7, one of the importin β family members, is another host cellular protein that is relevant to the nuclear import of the HIV-1 PIC (9). However, recent experiments with RNA interference technology have shown that importin 7 deficiency does not alter the efficiency of HIV-1 and simian immunodeficiency virus cDNA synthesis, nuclear translocation, or infection, suggesting that importin 7 is dispensable for HIV-1 infection (49). This result clearly demonstrates that importin 7 is not important for HIV-1 nuclear import in macrophages and that HIV-1 and simian immunodeficiency virus nuclear transport may involve other karyopherins or another unconventional mechanism. In this regard, the atypical importin α-dependent nuclear import of Vpr may be considered a candidate mechanism for transport of the HIV-1 PIC.

Binding of Vpr to importin α is a promising target for blocking HIV-1 replication. We demonstrated that after entering the host cell, the mutant αLA virus and L67P virus (15) may be restricted at the nuclear import step. We have speculated that Vpr nuclear import, mediated by its interaction with importin α, could play an important role in efficient HIV-1 infection, not only in monocyte-derived macrophages but also in activated $CD4^+$ T cells (15). A more complete understanding of this novel nuclear import mechanism will require a detailed investigation of the interaction between the αH1 domain of Vpr and the C-terminal region of importin α that leads to nuclear import. The interaction between Vpr and importin α may be a potential target for an antiviral agent that inhibits the nuclear entry step.

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