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). 3) Vpr interacts with importin α through two α helical domains, αH1 and αH3, which play distinct roles in nuclear import (18, 19). 4) Vpr interacts with the C-terminal region of importin α, and this interaction is required for nuclear entry (19). 5) The nuclear import of Vpr is promoted by the three major isoforms of importin α (Fig. 2). It has recently been reported that importin α alone can carry CaMKIV into the nucleus without utilizing importin β (23). Similarly to 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 substituted with Ala in 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 G2-arrest and apoptosis-promoting activities, to the halves of wile 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 our present study, Vpr appears 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, yeast 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, α1, α2 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 50-85% amino-acid identity, all isoforms interact via their IBB and C-terminal domains with importin β and cellular apoptosis susceptibility gene product (CAS) (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 non-classical 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 level and extent of reverse transcription is similar in monocytes and differentiated macrophages after infection with the HIV-based vector, whereas there are no detectable 2LTR 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 demonstrate that siRNA depletion of importin α from cytoplasmic extracts of HeLa cell markedly decreases Vpr nuclear import. Thus, we can conclude that importin α is required for the nuclear import of Vpr. Finally, experimental infection with virus encoding a Vpr mutant protein that cannot bind importin α shows 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 importin 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 previously investigated in human primary PBMC. In the present study, we demonstrate 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 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 α4, α6 and α7 express in monocytes, macrophages, resting $CD4^+$ T cells and activated $CD4^+$ T cells are targets of HIV-1 infection.

Importin 7, one of importin β family, 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 (SIV) 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 SIV 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 to be a candidate mechanism for transport of the HIV-1 PIC.

The binding of Vpr to importin α is a promising target for blocking HIV-1 replication. Herein, we demonstrate that, after entering the host cell, the mutant αLA virus 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.

ACKNOWLEDGMENTS

We thank Dr. Akio Adachi for kindly providing HIV-1 pNL432 and pNF462. We

thank Dr. Atushi Miyawaki for critical comments on imaging analysis.

This work was supported, in part, by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan (Research on HIV/AIDS 16150301), by Grants-in-Aid for Scientific Research on Priority Areas (1402113, 15019115, and 16017304), by a Grant-in-Aid for Exploratory Research (18659135) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, by a President's Special Research Grant from RIKEN, and by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

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FIGURE LEGENDS

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.

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 hr at 4°C, the bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. The positions of the three importin α isoforms are indicated.

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 RNA was extracted from monocytes and differentiated primary macrophages, and quantitative RT-PCR analysis was performed on the LightCycler system, using specific primers for each importin α isoform. Bars represent the mean values and standard errors from 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 importin 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.

FIG. 4. Importin α depletion prevents the nuclear import of Vpr in macrophages. (A) Lysates of differentiated primary macrophages were treated with MAb against importin α1 or 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 importin 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.

FIG. 5. The siRNA induced down regulation of importin α isoforms prevents the nuclear import of Vpr. (A) Twice of the siRNAs transfection corresponding to importins α1, α3, α5, β, or HPRT specific siRNA as a positive control, or a nonspecific siRNA as a negative control with HeLa cells were carried out. The cytoplasmic extracts were prepared from HeLa cells transfected with siRNA. Transfection efficiency of siRNA was determined by using Western blotting with against importin α1, α3, α5/7 and β specific antibodies. The positions of the 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, with containing 100 μg proteins of cytoplasmic extract prepared from HeLa cells transfected with siRNA. After fixation, cells were analyzed by confocal laser scanning microscopy.

Bar=20 μm.

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, GST-tagged GFP, GST-N17C74-GFP, GST-αH1-GFP, or GST-αLA/αH1-GFP, and incubated with 100 pmol recombinant importin α (top), 100 μg cytoplasmic extract from HeLa cells (middle), or none as the control (bottom). The proteins recovered from the beads were subjected to Western blotting with a MAb against importin α1. The positions of importin α are indicated. (C) Digitonin-permeabilized HeLa cells were incubated with 1 μM GST- and GFP-tagged N17C74 and 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 and αLA/N17C74 were 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 *vpr*-deficient form (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 (△), or a *vpr*-deficient form (◇), 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.

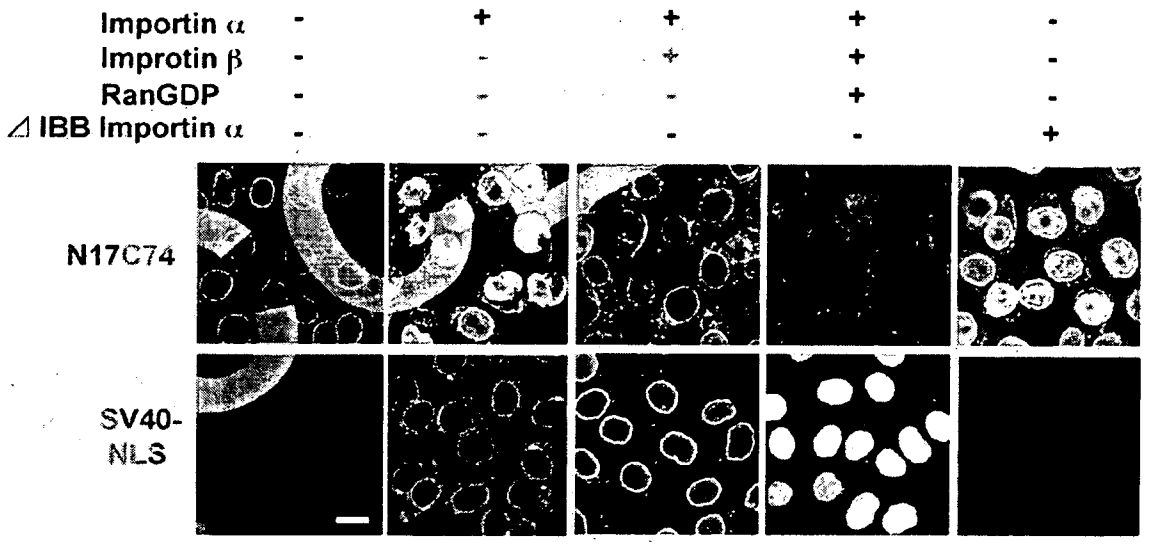

Fig.1

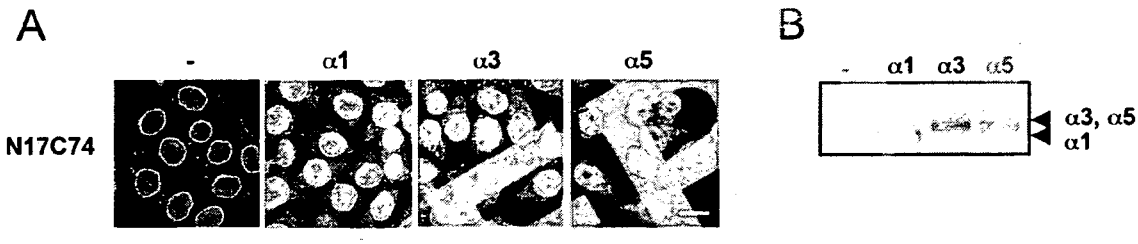

Fig.2

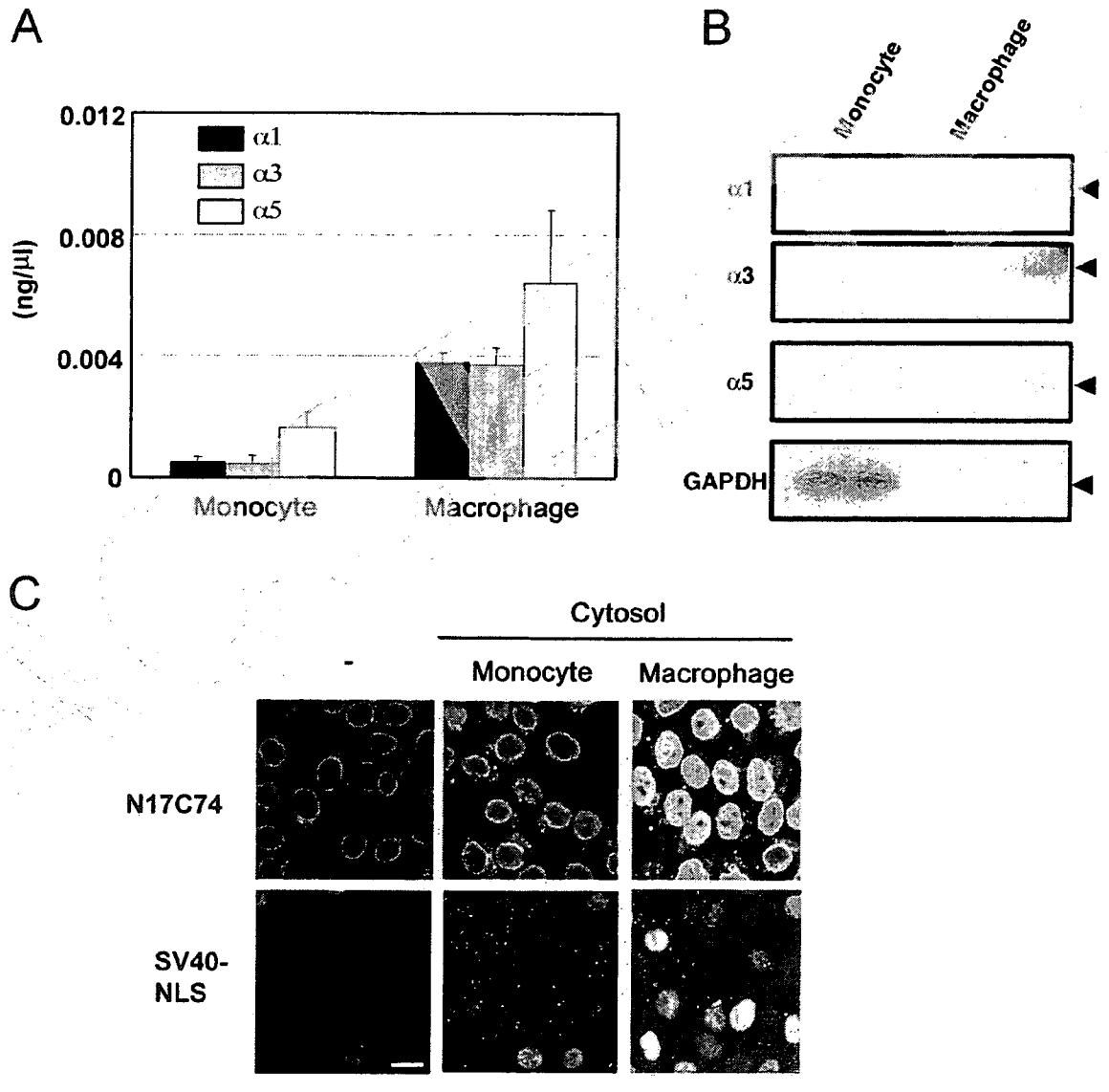

Fig.3