Vpr is localized in the first and third α-helixes [24]. Interestingly, it has been postulated that Vpr transduces proteins as well as plasmid DNA into cells [25,26]. The activity is energy-independent and requires no cellular receptors [27]. A Vpr-derived peptide consisting of amino acids 52–96 has two biological properties that facilitate efficient gene expression: the arginine-rich stretch present in the carboxy (C)-terminal region, at amino acids 80–96, is required for the interaction with plasmid DNA and the third α-helix, at amino acids 52–70, enables trafficking of plasmid DNA through endosomes to the cytoplasm [28].

Recently, we identified a sequence corresponding to amino acids 52–78 (C45D18) as having protein transduction activity (Fig. 1A) [29]. When recombinant proteins are conjugated with C45D18 and added to the culture medium, they are quickly transported to the nucleus. This nuclear trafficking activity of C45D18 is also effective in resting cells, and C45D18-conjugated proteins were incorporated into most cells that were serum-starved during culture, as well as into peripheral blood mononuclear cells. This led us to hypothesize that C45D18 can be used to improve non-viral expression systems applicable to resting cells.

In this study, we combined C45D18 with a non-viral vector system, a cationic polymer named star vector (SV), which is a chemically synthesized gene transfer vector. SV is a nano-structured, hyperbranched, cationic star polymer with highly efficient transduction activity [30–33]. We found that C45D18-SV could induce efficient gene expression in both chemically differentiated macrophages and monocyte-derived macrophages (MDMs). We present the results of an analysis of the expression level of an exogenous gene and incorporated plasmid DNA. We also discuss the mechanism of the improved gene transduction using C45D18 and future applications of the peptide.

Materials and methods

Cell culture and chemicals. HeLa and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MI). THP-1 cells (Riken Cell Bank, Tsukuba, Japan) were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) supplemented with 10% of FCS. To prepare resting cells, HT1080 cells were cultured for 3 days in 0.1% FCS medium. To differentiate THP-1, aliquots of 1×10^5 cells were plated onto each well of a poly-D-lysine-coated 6-well plate (Beckton–Dickinson, Benford, MA) and treated for 2 days with 5×10^{-8} M phorbol myristate acetate (PMA) (Sigma). The expression of Mac-1, a macrophage marker, was checked with a specific antibody (BD Pharmingen, San Diego, CA). To prepare human MDMs, peripheral blood mononuclear cells obtained from healthy humans were cultured for 4–7 days in the presence of 100 ng/ml of macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) [34]. The expression of Mac-1, a macrophage marker, became positive after 2 days of culture (data not shown). For the transduction experiments, MDMs were cultured for an additional 4 days without M-CSF, and then the adherent cells were trypsinized and re-plated at a concentration of 2×10^5 cells per well in a 6-well plate.

Synthesis of hexakis(N,N-diethyldithiocarbamyl(oligo(4-aminostyrene)-block-poly(3-(N,N-dimethylamino)propylacrylamide)))methylbenzene (6-star-PDMAPAAm-b-OAS) (SV). Hexakis(bromomethyl)benzene and 4-aminostyrene (AS) (Sigma–Aldrich, Milwaukee, WI), sodium *N,N*-diethyldithiocarbamate (Wako Pure Chemical, Osaka, Japan), and 3-(*N,N*-dimethylamino)propylacrylamide (DMAPAAm) (Tokyo Kasei Kogyo, Tokyo, Japan) were purchased. Other chemical reagents were from Wako. A cationic star polymer with six poly[(*N,N*-dimethylamino)propylacrylamide] (PDMAPAAm) chains terminated with oligo(4-aminostyre) (OAS) per molecule (6-*star*-PDMAPAAm-*b*-OAS) was synthesized by iniferter-based photo-living radical polymerization of DMAPAAm and then AS from hexakis(*N,N*-diethyldithiocarbamylmethyl)benzene [32,33]. Briefly, hexakis(*N,N*-diethyldithiocarbamylmethyl)benzene was synthesized with reaction of hexakis(bromomethyl)benzene and sodium *N,N*-diethyldithiocarbamate, and crystallized from a chloroform–*n*-hexane solution. To synthesize 6-star-PDMAPAAm, the mixture of hexakis(*N,N*-diethyldithiocarbamylmethyl)benzene and DMAPAAm was irradiated for 30 min with a 200 W high pressure mercury lamp (Spot Cure, Ushio, Tokyo, Japan), concentrated, and dissolved in a small amount of methanol. The precipitate, obtained by the addition to ether (500 ml), was separated by filtration. Reprecipitation was performed in a methanol–ether system three times. The last precipitate was dried under vacuum to give 6-*star*-PDMAPAAm. To produce 6-star-PDMAPAAm-*b*-OAS, the mixture of 6-*star*-PDMAPAAm and AS was irradiated for 30 min under the above-mentioned conditions. The reaction mixture was concentrated and precipitated in 500 ml ether. Reprecipitation was carried out in a chloroform–ether system three times. The last precipitate was dried under vacuum to give 6-*star*-PDMAPAAm-*b*-OAS. The total molecular weight was about 18,300, which was estimated from GPC and ^{1}H NMR spectra. About 17.3 molecules of DMAPAAm unit and 1.4 molecules of AS unit were introduced to each terminal of all the six branch chains in the 6-*star*-PDMAPAAm-*b*-OAS.

Peptide synthesis and conjugation with SV. C45D18, other Vpr-derived peptides (shown in Fig. 1A), and Tat-derived peptide composed of GYGRKKRRQRRRGGC (single-letter amino acid code) [29] were synthesized chemically (Wako). Each peptide contains cysteine in C-terminal region and its SH-residue is used for conjugation to SV compound. Approximately 1 mg of SV was suspended in 1 ml of 10 mM phosphate buffer (pH 7.0) and added to 0.1 mM *N*-[*e*-maleimidocaproyloxy]succinimide ester (Dojindo Lab. Kumamoto, Japan). After 30 min at room temperature, each peptide was added and further incubated for 3 h at room temperature. The free peptides were removed by dialysis against phosphate-buffered saline overnight. The molar ratio of the peptide to SV was usually 3:1.

Cell cycle analysis. Cells were treated for 30 min, 1 or 2 days with 10 μM bromodeoxyuridine (BrdU) (Sigma). After fixation in 70% ice-cold ethanol, the cells were treated with an anti-BrdU antibody (Beckton–Dickinson) and then detected with Cy3-labelled antibody to mouse IgG (Molecular Probes, Eugene, OR).

Transfection and analysis of transduced genes. pLuc/EGFP constructed with pGL3 (Promega, Madison, WI) and pIRES2-EGFP (Clontech, Mountain View, CA) was used as a reporter construct. First, we examined complex formation involving the plasmid DNA and the peptides, SV and C45D18-SV. We incubated 250 ng of plasmid DNA with various amounts of peptides or other compounds in 150 μl Opti-MEM (Invitrogen). After 30 min at room temperature, the aliquots were loaded onto an agarose gel to determine the minimum amount of the compound required to completely neutralize the anionic charge of the DNA and then added to cell culture. After 48 h, luciferase assay was carried out (PicaGene, Toyoink, Tokyo, Japan). The protein concentration was measured by using a Bradford system (Bio-Rad, Hercules, CA) and the relative right units (RLUs) were normalized using the protein concentration.

To examine the level of exogenous gene expression, PCR analysis was carried out on reverse transcribed mRNA of the *GFP* gene. After transfection, mRNA was extracted using RNeasy (Invitrogen), and cDNA was synthesized with oligo(dT) (Qiagen, Hilden, Germany), and then amplified using an EX-Taq polymerase (TaKaRa, Shiga, Japan). To amplify the *β-actin* or *EGFP* genes, we used the respective forward and reverse primers 5′-TGAACCCCAAGGCCAACCGC-3′ and 5′-TTGTGCTGGGTGCCAGGGCA-3′ for *β-actin* or 5′-ATGGTGAGCAAGGGGCGA GGA-3′ and 5′-TTACTTGTACAGCTCGTCC-3′ (Hokkaido System Science, Sapporo, Japan) for *EGFP*. The amplified DNA was applied on an agarose gel, and the electrophoresed DNA was stained with Vistra Green

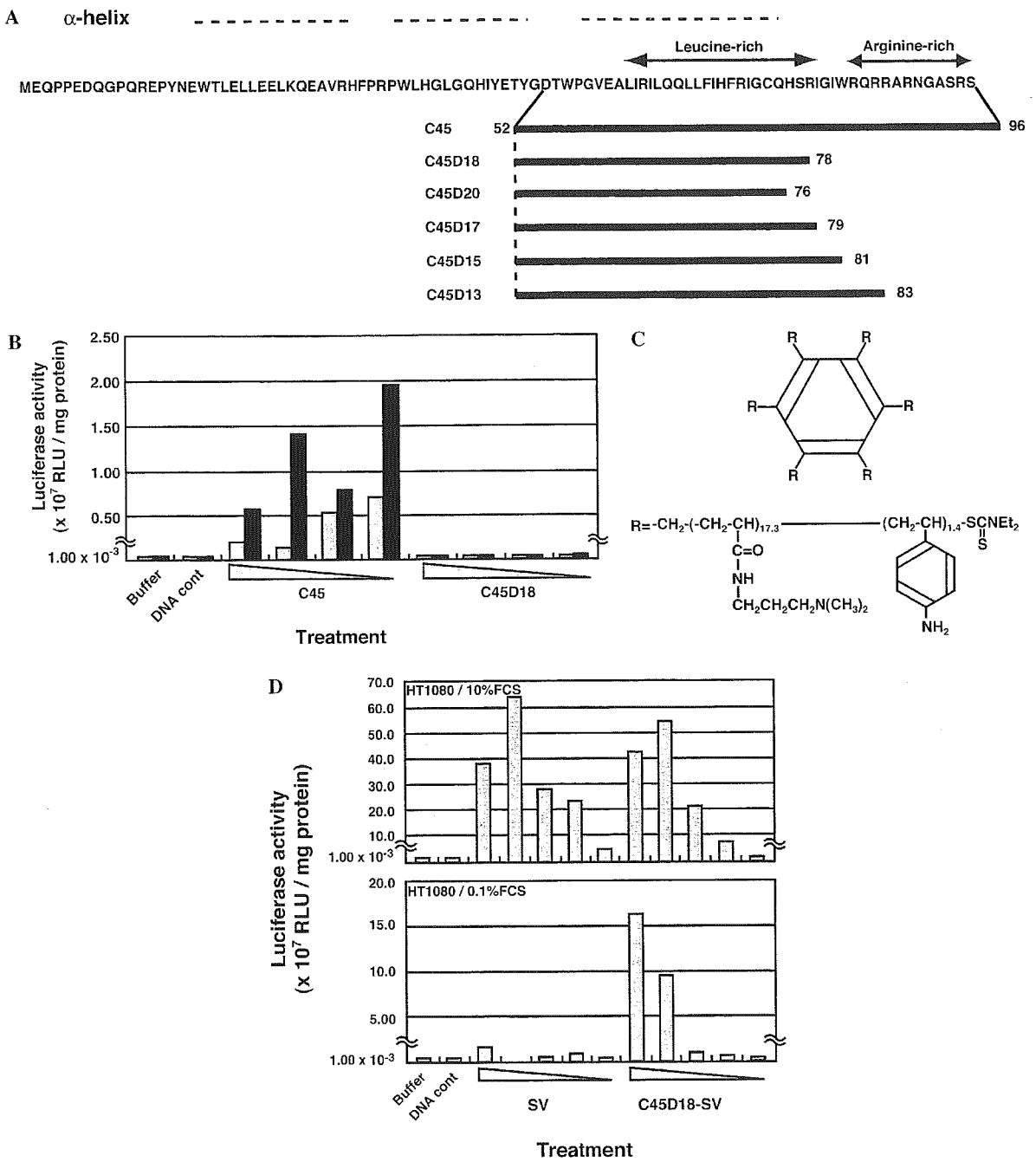

Fig. 1. Gene transduction by C45D18. (A) The amino acid sequences of the peptides used in this study. Amino acids are written using single-letter code. The α-helixes and arginine-rich stretch are also shown. Regions corresponding to six Vpr-derived peptides are depicted. (B) Gene transduction by C45 and C45D18 into growing cells. The reporter plasmid DNA, pLuc/EGFP, was mixed with C45 or C45D18, and was then added to HeLa (gray columns) or THP-1 (black columns) cells. Various concentrations of the peptides, ranging from 400 to 15 μM, were used. One representative result out of three independent experiments is shown. Luciferase activity is shown as relative light units (RLUs)/mg protein. (C) Schematic structure of SV. The SV used in this study has six branches (shown by "R") (21). The arrow indicates the amino residue that is used for conjugation with various peptides. (D) Efficient gene transduction by C45D18 into serum-starved cells. The gene transduction efficiencies using C45D18-SV and SV were compared. The HT1080 cells were cultured for 3 days in the presence of 0.1% FCS (lower panel) or under normal conditions (upper panel) and were then used for the experiments. The respective frequencies of BrdU-positive cells in the population were 9% and 43% for serum-starved cells and those cultured in the presence of 10% FCS, respectively. For each sample, 250 ng of plasmid DNA was mixed with 45–1.4 μM of the compounds.

(Amersham Biosciences, Piscataway, NJ). Then, the signal intensity of each band was measured using FX-PRO PLUS (Bio-Rad).

To analyze the plasmid DNAs incorporated in the nuclei, an aliquot of plasmid DNA was labeled with Cy3-dCTP (Amersham Biosciences), mixed with the same amount of plasmid DNA, and then used for transfection. The incorporated plasmid DNA was visualized using laser scanning microscopy (Bio-Rad). Cells positive for DNA aggregates that were larger than 2 μm were counted.

Statistical analysis. Statistical significance was evaluated using Student's *t* test.

Results

Gene transduction with C45 and C45D18

First, we compared the gene transduction activity of two peptides, C45 and C45D18 (Fig. 1A). Based on the results of a gel-shift assay to monitor the lipoplex formation of pLuc/EGFP (250 ng) with C45 (see Materials and methods), we used 15, 45, 135, and 400 μM of the peptide for the experiment. The C45 and an equivalent amount of C45D18 were incubated with plasmid DNA for 30 min at room temperature and were then added to cultures of HeLa and THP-1 cells. Consistent with previous reports [35,36], C45 was effective for gene transduction into these cells (Fig. 1B). By contrast, C45D18 did not show any transduction activity.

C45D18 facilitates gene transduction into resting cells

We previously reported that recombinant proteins, when conjugated with C45D18, could be transported into the nuclei of resting cells [29]. This finding led us to postulate that C45D18 could facilitate gene expression in resting cells, if an appropriate vehicle for the DNA were selected. To prove this, C45D18 was conjugated to SV (C45D18-SV; Fig. 1C), and we compared the efficiency of gene transduction using C45D18-SV with that using SV alone. Although the gene expression in growing cells was equivalent with both agents (Fig. 1D, upper panel), C45D18-SV resulted in more efficient gene expression in cells that had been cultured in the presence of 0.1% FCS (Fig. 1D, lower panel). Analysis of the cell cycle, judged using incorporated BrdU, revealed that the number of cells in S phase was decreased remarkably in the populations cultured in 0.1% FCS (9%) as compared with those cultured in 10% FCS (43%).

C45D18-SV increased the gene transfer to human macrophages

To obtain stronger evidence that C45D18 facilitates gene transduction into resting cells, we used THP-1 cells that had been treated with PMA. THP-1 is a non-adherent human monocytic leukemia cell line that acquires the macrophage-phenotype when cultured in the presence of PMA. Within 2 days after PMA treatment, THP-1 cells became adherent and positive for Mac-1, a macrophage-specific marker (data not shown). Then, we tested the efficacy of gene transduction using C45D18-SV. As shown in Fig. 2A, luciferase expression with C45D18-SV transduction was more than 40 times that with SV alone ($p < 0.05$). To examine the expression level of the exogenous gene, we amplified the *GFP* mRNA from transfectants treated with SV alone or with C45D18-SV and compared the intensities of the amplified DNA. As shown in Fig. 2B, the level of gene expression was increased strikingly in the cells treated with C45D18-SV (lane 2). Pulse-labeling with BrdU followed by detection with anti-BrdU

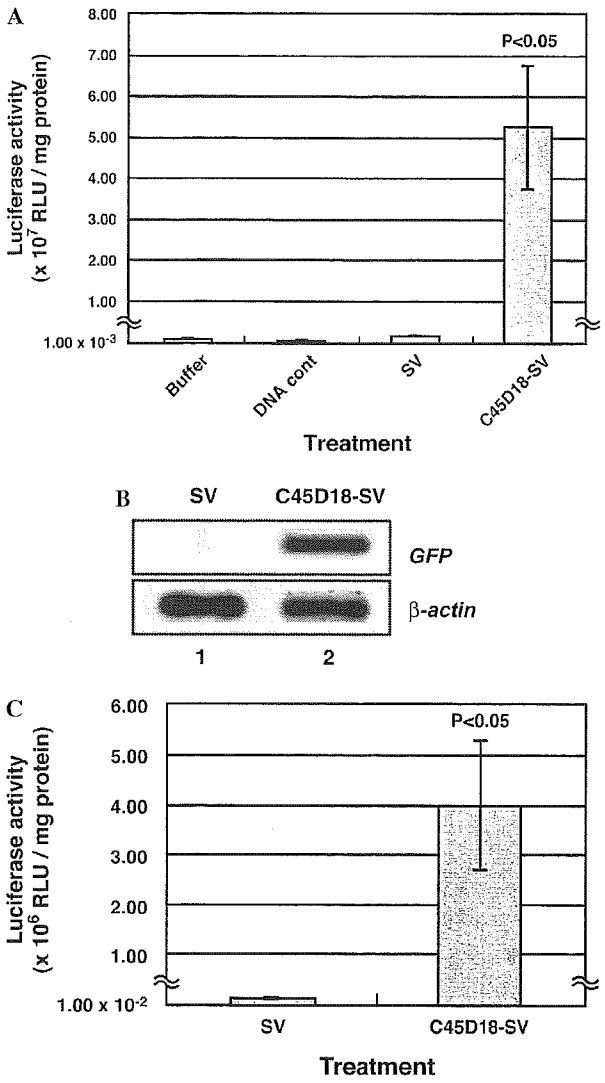

Fig. 2. Increased gene expression using C45D18-SV in resting macrophages. (A) The effects of C45D18-SV on chemically differentiated macrophages. THP-1 cells, after treatment for 2 days with PMA, were used for the transfection experiments. The experiments were carried out in triplicate, and the mean numbers and standard deviation were calculated. BrdU incorporation indicated that less than 1% of the cells were at S phase. Even with continuous exposure to BrdU for 2 days after transfection, less than 1% of the cells were BrdU-positive. (B) The expression of the exogenous gene using C45D18-SV in resting macrophages. RT-PCR analysis was performed using THP-1 cells treated with PMA and then transfected. As an internal control, β-actin mRNA was amplified. The results of the RT-PCR analysis of cells transduced using SV (lane 1) and C45D18-SV (lane 2) are shown. (C) Gene transduction into human macrophages. MDMs were prepared from healthy humans by culturing peripheral blood mononuclear cells for 6 days in the presence of 100 ng/ml M-CSF. Then, the cells were deprived of M-CSF for 4–5 days and subjected to experiments in triplicate. The mean value and standard deviation are shown, along with a representative result of three independent experiments. Immunohistochemical analyses of pulse-labeled BrdU and Mac-1 indicate that the prepared cells were resting macrophages (data not shown).

antibody revealed that less than 1% of PMA-treated THP-1 cells were positive for BrdU incorporation (data not shown), whereas about 30% of the untreated THP-1 cells were positive for BrdU incorporation. Moreover, with

continuous exposure to BrdU for 2 days after transfection, only 0.6% of the PMA-treated cells were labeled with BrdU (data not shown). These results indicate that THP-1 cells stop growing within 2 days after treatment with PMA and that C45D18-SV facilitates gene expression in resting macrophages.

Next, we focused on gene transduction into human MDMs using C45D18-SV. To test the efficacy of C45D18-SV, human macrophages were prepared by culturing peripheral blood mononuclear cells for 6 days in the presence of 100 ng/ml M-CSF. After cell expansion, the M-CSF was removed from the culture, and the culture was continued for another 4–5 days, which caused the expanded MDMs to stop growing (data not shown). Using these cells, we investigated the efficacy of C45D18. As shown in Fig. 2C, the transduction efficiency with C45D18-SV was significantly greater than that with SV alone ($p < 0.05$). We repeated the same experiments three times and obtained essentially the same statistically significant results.

C45D18-SV is the best molecule for gene transduction into macrophages

To determine the best Vpr-derived peptide for gene transfer into macrophages, several peptides were synthesized (Fig. 1A) and conjugated to SV at a molar ratio of 3:1. First, we compared the activities of C45 and C45D18. As shown in Table 1, C45-SV was much less potent than C45D18-SV in resting macrophages ($p < 0.01$), which is in contrast to the result, shown in Fig. 1B, that C45 induced much better gene expression in growing cells than did C45D18.

Next, we compared the activity of C45D18 with those of four other peptides. Based on several independent experiments, we estimated that the gene transduction efficiencies with C45D17, C45D15, C45D13, and C45D20 relative to that with C45D18 (1.0) were 0.25, 0.30, 0.45, and 0.32, respectively (Fig. 3A). Therefore, C45D18 is the minimum sequence giving the best efficiency of gene expression in resting macrophages.

Protein transduction activity has been well documented for Tat-derived peptide. Although the gene transduction activity of Tat has not been reported, we compared the gene transduction efficiency of Tat and C45D18. As shown in Fig. 3B, Tat-conjugated SV did not result in significant expression of the exogenous gene, whereas C45D18-SV reproducibly induced high expression from the plasmid DNA in resting macrophages.

Table 1
Gene-transducing activity of C45D18 and C45 to resting cells

Samples	RLU ($\times 10^{-5}$)/mg protein	Fold increase[b]	t test
Buffer	1.8 ± 0.4[a]	1	
DNA alone	2.7 ± 1.8	1.5	
SV alone	4.7 ± 2.0	2.7	
C45-SV	11.4 ± 6.4	6.5	$p < 0.01$
C45D18-SV	96.6 ± 10.1	55.2	

[a] The relative right units (RLUs) were normalized using the protein concentration. The mean values and standard deviations were calculated from triplicate samples. Difference between luciferase activity obtained by C45-SV and C45D18-SV was statistically significant ($p < 0.01$).

[b] The fold increase was estimated using data for the buffer as control.

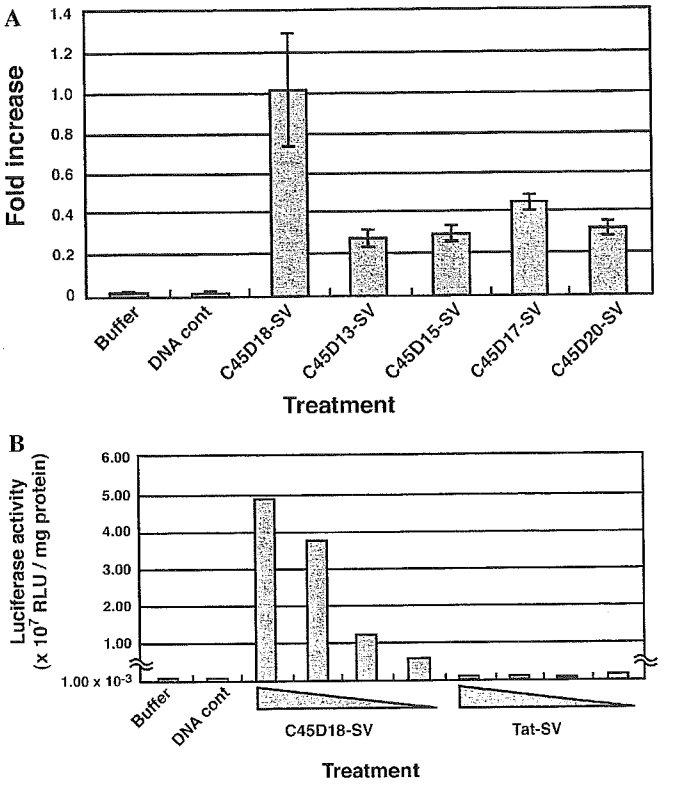

Fig. 3. C45D18 is the best molecule for efficient gene expression in resting macrophages. (A) Comparison of the gene transduction activities among C45D18 and four Vpr-derived peptides (see Fig. 1A). Each peptide was conjugated to SV at a molar ratio of 3:1, and the efficiency of gene expression in resting macrophages was analyzed. For each peptide, 17 μM peptide was reacted with 250 ng pLuc/EGFP. Three independent experiments were performed, and the mean and standard deviation were calculated. The activity of each peptide is given as a relative efficiency by defining the activity of C45D18-SV as 1.0. The actual luciferase activity obtained with C45D18-SV was $5.2 \pm 1.6 \times 10^7$ RLU/mg protein. (B) The effects of Tat-derived peptide on gene transfer into resting macrophages. Tat-derived peptide was conjugated to SV at a molar ratio of 3:1, and then the efficiency of gene expression in resting macrophages was examined. The maximum dose of each conjugated compound was 12 μM with 250 ng plasmid DNA. Each compound was serially diluted to 6, 3, and 1.5 μM, and the compound was reacted with the reporter plasmid DNA.

Condensed localization of exogenous DNA in the nucleus using C45D18-SV

To characterize the DNA incorporated in the nucleus by C45D18, we investigated the plasmid DNA in resting macrophages after transfection. For this purpose, an aliquot of plasmid DNA was labeled with Cy3-dCTP, mixed with an equal amount of unlabeled DNA, and used for the transfection experiment. Surprisingly, we observed the presence of plasmid DNA as large dots in the nucleus when C45D18-SV was used as the vector (Fig. 4A, right panels),

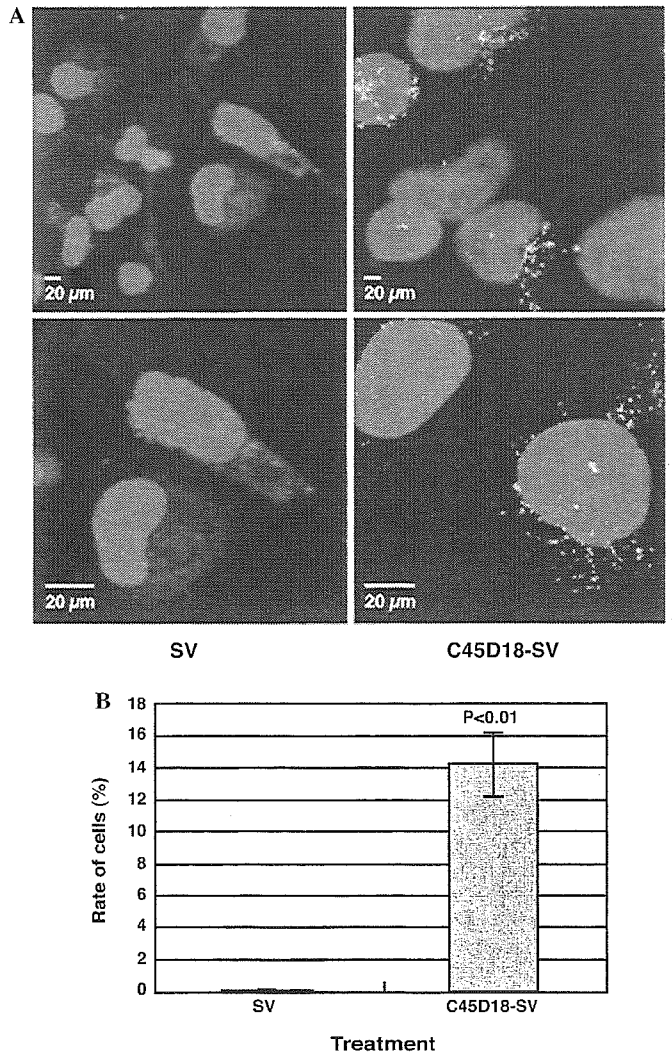

Fig. 4. Analysis of plasmid DNA introduced using C45D18-SV. (A) The aggregation of exogenous DNA in cells transduced with C45D18-SV. Plasmid DNA incorporated with SV (left panels) or C45D18-SV (right panels) was visualized using laser scanning microscopy. A small amount of plasmid DNA that had been pre-labeled with Cy3 was mixed with an equal amount of unlabeled plasmid DNA. The lower panels show enlarged cells. Plasmid DNA and nuclear DNA are shown in red and blue, respectively. The scale bar indicates 20 μm. (B) Increased number of cells with aggregated plasmid DNA. The number of cells containing large dots of plasmid DNA (>2 μm) was counted and plotted. At least 100 cells were counted per microscopic field. The mean and standard deviation were calculated using data obtained from three independent experiments. The difference between cells transduced using SV and those transduced using C45D18-SV was statistically significant ($p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

whereas fine dots of DNA were present in the cells transfected with SV alone (Fig. 4B, left panels). We counted the number of cells that contained labeled plasmid DNA dots larger than 2 μm. As shown in Fig. 4B, C45D18-SV dramatically increased the number of cells containing large dots of plasmid DNA; about 15% of the cells transduced with C45D18-SV contained large dots of aggregated plasmid DNA ($p < 0.01$). PCR analysis indicated that the copy number of plasmid DNA in cells treated with C45D18-SV was at most 1.7-fold that in cells treated with SV (data not shown).

Discussion

C45D18-SV is an effective, novel non-viral vector for gene transduction into human macrophages

We showed that C45D18 facilitates gene transduction into resting macrophages. In this work, we used SV as a vehicle for the plasmid DNA. SV is a synthetic cationic polymer used as a gene transfer vector [30]. It was previously demonstrated that SV can induce a gene transduction level 10-fold that induced with polyethyleneimine (PEI), which is one of the best agents popularly used for gene transfer [30]. Although SV by itself was not effective for introducing genes into chemically differentiated macrophages, C45D18-SV could promote the expression of exogenous genes at a significantly higher level (Figs. 2A and B). Furthermore, C45D18-SV could facilitate the expression of an exogenous gene in quiescent human MDMs (Fig. 2C). These results are consistent with our previous report that recombinant proteins conjugated with C45D18 could be transported into the nuclei of resting cells [29]. Based on these observations, we postulate that the C45D18-SV system will be effective for other types of resting cells. This possibility is now under investigation.

Of the peptides with protein transduction activity, it has been reported that Tat can transduce recombinant proteins, which are expressed as chimeric forms with Tat-derived peptide. In this work, we also tested the activity of the Tat-derived peptide, but Tat-SV induced few exogenous genes in resting macrophages (Fig. 3B). Therefore, C45D18 is the best candidate molecule for circumventing the difficulties of non-viral vector systems applied to resting cells.

Differential activity of C45D18 and C45

Interestingly, we observed a functional difference between C45 and C45D18. As reported [35,36], C45 could express plasmid DNA in growing cells (Fig. 1B), whereas C45D18 could not cause transduction alone; however, after conjugation to a cationic polymer, C45D18 showed unique activity, enabling gene expression in resting macrophages. By contrast, C45-SV was much less effective for gene transduction into resting cells (Table 1).

Vpr binds DNA through its C-terminal region [37]. C45D18 lacks the C-terminal region that is required for DNA binding, which explains why C45D18 alone could not cause gene expression. With C45D18 conjugated to SV, the cationic moiety of SV complemented the DNA binding property, restoring the nuclear trafficking activity of C45D18, which led to the transduction of genes into resting macrophages. By contrast, the conjugation of C45

to SV decreased its ability to cause gene expression in resting cells (Table 1). Both C45D18 and C45 were conjugated to SV through the cysteine residue at amino acid 76 of Vpr (see Materials and methods, and Fig. 1A). One possible explanation as to why C45 activity was decreased after conjugation to SV is that the interaction between the cationic moieties of C45 and SV interferes with the properties of the leucine/isoleucine-rich domain (LR-domain) of Vpr [23] (Fig. 1A). The LR-domain (amino acids 60–81) was reported to be required for nuclear trafficking of Vpr, and point mutations at amino acids 64, 67, 71, and 76 significantly reduced the Vpr activity of nuclear trafficking [24]. It would be important to conjugate C45D18 to other compounds through the third position of its C-terminal region to keep the amino half of C45D18 fully functional. In this context, it is likely that the nuclear trafficking activity of C45D18, if conjugated appropriately, would contribute to non-viral vector systems other than SV.

Effects of C45D18 on transduced plasmid DNA

Strikingly, the incorporated DNA aggregated in the nucleus when C45D18-SV was used as a transfer vector (Fig. 4A). About 15% of the cells were positive for large dots of plasmid DNA (>2 μm). By contrast, we detected few such cells after transduction by SV alone. It is still not known whether aggregated molecules are effective for gene expression. Although RT-PCR demonstrated that C45D18-SV strongly induced the expression of an exogenous gene (Fig. 2B), PCR analysis of the transduced DNA revealed that C45D18-SV did not dramatically increase the copy number of the exogenous gene, but gave at most 1.7 times the plasmid DNA produced with SV. These data suggest that C45D18-SV increased the local concentration of the plasmid DNA, resulting in increased expression of the exogenous gene.

The aggregation of plasmid DNA by C45D18 may impair the expression of the exogenous gene. When genes are transduced using a non-viral gene transfer system, the plasmid DNA must be released from the gene-transducing agent for efficient gene expression [38]. If the plasmid DNA transduced into nucleus is surrounded by the gene transfer agent, for example, by PEI, transcription cannot effectively occur [39]. When we examined the numbers of cells with exogenous gene expression, less than 1% of total cells were positive for GFP expression. Even in an immunohistochemical analysis with anti-GFP antibody, we observed that GFP-positive cells, indicating the transducing efficiency of the exogenous gene, increased to 0.5% from 0.1% on using C45D18-SV compared with SV alone. The data indicate that C45D18 increased the number of cells that are positive for expression of exogenous DNA, but most of the plasmid DNA transported into the nucleus unfortunately does not work well as a template for transcription. It is still necessary to develop a system in which exogenous genes transported to the nucleus by C45D18 are effectively released for favorable gene expression.

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compared with four in the experienced group. Four of these five patients had HCV co-infection. Two events arose after one month of treatment and the other three after a year, confirming the multifaceted mechanisms causing this toxicity. In all these cases the treatment had to be stopped, and the patients regressed.

To the best of our knowledge, this study comprises the biggest series to date of patients treated with lopinavir/ritonavir and followed prospectively outside clinical trials. In addition, this HIV-positive population had a high prevalence of co-infection with hepatitis viruses.

The frequency of hepatotoxicity was actually low, unlike in other studies. This might partly be the result of methodological differences, reflecting how the data were collected. Retrospective studies can suffer major selection bias. Gonzalez-Requena *et al.* [11] also reported a low incidence of adverse events, but their case series was small and was followed up for not more than one year.

In conclusion, the present study found that lopinavir/ritonavir caused only limited hepatic toxicity in this population of HIV-positive patients with a high prevalence of co-infection with hepatitis B virus or HCV.

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Premature sister chromatid separation in HIV-1-infected peripheral blood lymphocytes

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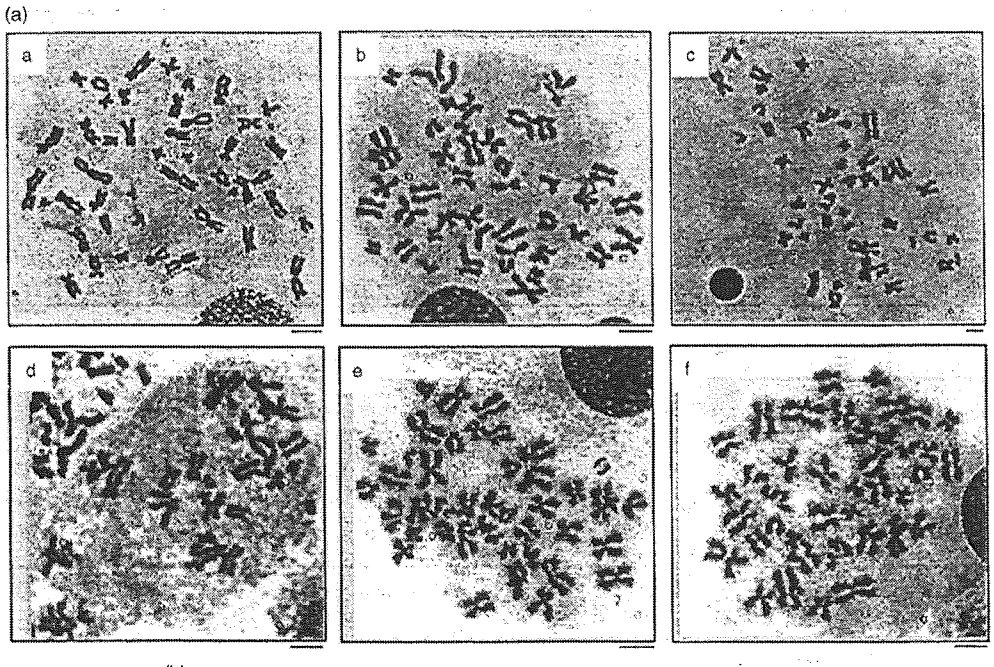
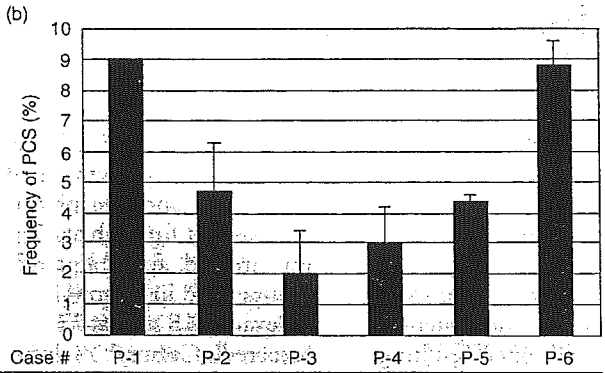
To investigate the mechanism of aneuploidy that is frequently observed in AIDS, we examined premature sister chromatid separation (PCS), a sign of genomic instability, in peripheral blood cells of HIV-1-infected individuals. PCS was found in all

six HIV-1 individuals at a high incidence. When peripheral blood cells from healthy volunteers were infected with HIV-1 *in vitro*, the incidence of PCS increased. This suggests that HIV-1 infection causes PCS and has the potential to induce aneuploidy.

Malignancy in HIV infection influences the prognosis of AIDS patients. These neoplasms are the result of various diseases that accompany immunodeficiency, such as co-infections with Epstein–Barr virus or human herpes virus 8 [1–4]. Besides these AIDS-defining cancers, several non-AIDS-defining cancers also occur at a higher incidence in HIV-infected individuals [5–9]. Moreover, it has been reported that HIV-1 itself is tumorigenic in immortalized B cells in nude mice [10,11]. These reports lead to the hypothesis that HIV-1 has the potential to induce neoplasms before AIDS develops.

Aneuploidy is a phenomenon of chromosome instability that is frequently reported in HIV-1-infected individuals





Case #	P-1	P-2	P-3	P-4	P-5	P-6
Total WBC	3800	5500	8400	5700	6100	2700
HIV-1 mRNA (copies/ml)	27,000	< 50	76	< 50	< 50	35,000

Fig. 1. Metaphase spreads of blood cells in HIV-1 infection. (a) Representative metaphase spreads of peripheral blood cells from HIV-1-infected individuals (b, c, d, e, and f are from cases nos. P-1, 2, 4, 5, and 6, respectively, see Fig. 1b). (a). (b) Frequency of premature sister chromatid separation (PCS). The frequency of PCS (black bar), and number of HIV-1 messenger RNA copies and total white blood cells (WBC) are shown. (c) Metaphase spreads of peripheral blood mononuclear cells (PBMC) from healthy volunteers. Representative metaphase spreads of PBMC from healthy volunteers with (a/+, b/+, and c/+) or without (a, b, and c) vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 infection are shown. (d) Aneuploidy in HIV-1-infected cells. Metaphase spreads from P-1, P-6 and from PBMC with VSV-G-pseudotyped HIV-1 infection were positive for aneuploidy with numbers of chromosomes of 85, 75 and 65, respectively. The scale bar represents 5 μm.

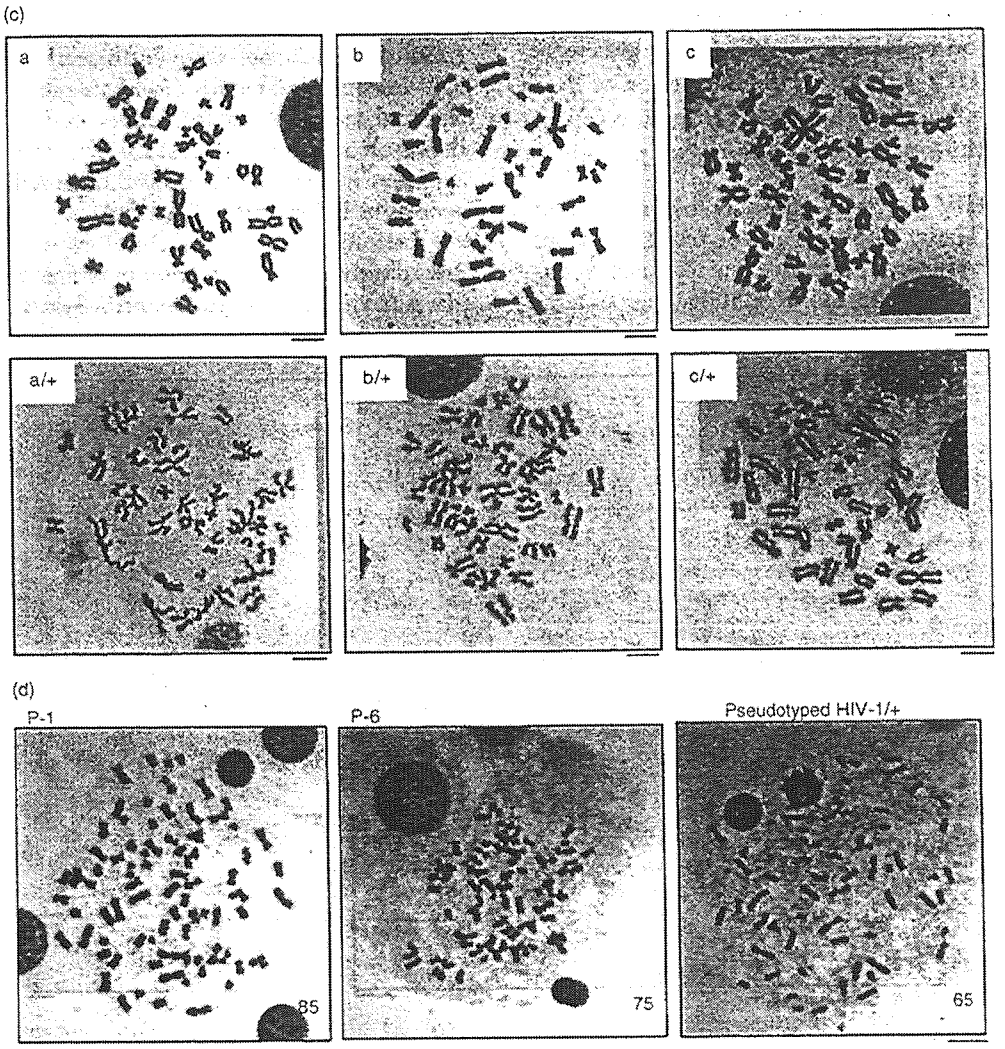


Fig. 1. *(continued)*

[12–14]. One of the major factors accelerating aneuploidy is thought to be abnormal chromatid separation [15–17]. At metaphase, paired sister chromatids are folded at the centric region until the onset of anaphase [18–22]. If the attachment of the sister chromatids is abolished before the onset of anaphase, premature sister chromatid separation (PCS) occurs. Subsequently, chromosome mis-segregation is induced, often resulting in aneuploidy [16,17]. PCS has been found in several clinical conditions, including aging, familial dominant inheritance [23–25], Roberts syndrome [26,27], cancer-prone syndrome mosaic variegated aneuploidy [28,29] and general tumours [30,31]. Note that all of these cases of PCS are associated with aneuploidy, indicating that a high PCS rate is a sign of chromosome instability. To investigate the cellular mechanism of HIV-1-related aneuploidy, we examined PCS in peripheral blood cells of HIV-1-infected individuals.

Peripheral blood was collected in sodium heparin (20 U/ml) from HIV-1-infected patients or healthy volunteers. We added 0.5 ml whole blood to 9.5 ml RPMI-1640 growth medium containing 10% fetal calf serum and 2% phytohemagglutinin M-form, and incubated it for 82 h at 37°C. Then colcemid (30 ng/ml) was treated for 2 h at 37°C. Recovered cells were resuspended in 75 mM potassium chloride and incubated for exactly 15 min at 37°C. To the cell suspension, freshly prepared Carnoy's solution (methanol : glacial acetic acid = 3 : 1) was added and mixed gently. After three changes of Carnoy's solution, a drop of the cell suspension was placed on a slide and air dried. Subsequently, the metaphase spread was stained with Giemsa.

Surprisingly, the HIV-1 patients examined showed PCS at high frequencies of 2.1 to 9.0% (mean ± standard deviation; 5.36 ± 2.92%; Fig. 1a, panels b–f and Fig. 1b). A high incidence of PCS was observed in HIV-1-infected individuals with high viral RNA copy numbers (Fig. 1b), in which total PCS was often observed (patient case no. 1 and no. 6; panels b and f). By contrast, peripheral blood mononuclear cells (PBMC) from healthy volunteers

showed normal attachments at the centromere (Fig. 1a, panel a), and PCS was detected in less than 2% (1.22 ± 0.48%).

We next clarified whether the PCS was attributable to HIV-1 infection. The PBMC (1.5×10^6) [32] were infected with vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 [33] at the concentration of 2 ng/ml of p24 Gag antigen of pseudotyped virus (multiplicity of infection at 0.007). They were incubated for 82 h in the presence of 2% phytohemagglutinin M-form, and metaphase spread was analysed as described above. All of the specimens from three volunteers showed an increased incidence of PCS after HIV-1 infection (Fig. 1c, lower panels), whereas PCS was barely detectable without infection (Fig. 1c, upper panels). The frequencies of PCS after HIV-1 infection in the three samples were 8.40 ± 1.09, 5.28 ± 1.40, and 7.34 ± 1.67, whereas the frequencies without infection were 1.26 ± 0.40, 0.72 ± 0.22, and 1.68 ± 0.86, respectively. Our present data suggest that HIV-1 infection is a primary factor inducing PCS.

In the patients' case, the frequency of PCS was positively correlated with the reduction in total white blood cells (Pearson product–moment correlation coefficient $r = 0.837$, $P < 0.01$; Fig. 1b) rather than CD4 positive lymphocytes ($r = 0.011$, $P > 0.05$). Although VSV-G-pseudotyped HIV-1 was infected to PBMC at a multiplicity of infection of 0.007 (0.7%), the average incidence of PCS with HIV-1 infection exceeded 7%. Taken together with the information that pseudotyped HIV-1 induces a single round of infection, these data suggest that PCS occurs not only in response to the infection itself but also as a result of the effects of other virus products or cellular proteins stimulated by HIV-1 infection.

Simultaneously, we found aneuploidy in hyperploid cells of HIV-1-infected individuals who had high viral loads and high PCS frequency (Fig. 1b and Fig. 1d, left and middle panels). We also found aneuploidy in PBMC with HIV-1 infection *in vitro* (Fig. 1d, right panel). By contrast, aneuploidy was not found in control PBMC. Although it remains to be determined whether PCS is directly related to neoplasms in AIDS, we speculate that a high incidence of PCS and constitutive virus infection augment the susceptibility of the cells to aneuploidy and may play a critical role in the development of AIDS-related neoplasms. It will be important to track the epidemiological and biological features of the incidence of PCS in HIV-1 infection.

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Nuclear trafficking of macromolecules by an oligopeptide derived from Vpr of human immunodeficiency virus type-1

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Abstract

Vpr, an accessory gene product of HIV-1, is incorporated into cells when added to the culture medium. Via such function Vpr has been shown to transduce a protein into cells that is expressed as a chimeric protein with Vpr. The domain required for protein transduction, however, remained to be clarified. Here we identified a sequence encompassing 52–78 amino acids of Vpr (C45D18) that enables nuclear trafficking of proteins. When chemically synthesized C45D18 was added to the culture medium of human cord blood mononuclear (CBMN) cells, most cells became positive for the incorporated C45D18. Furthermore, recombinant proteins conjugated with the C45D18 were efficiently transduced and transported to regions corresponding to the nucleus. Incorporation of C45D18-conjugated protein was observed within a few hours after addition of the protein, independent of cellular growth. Although it is well known that Tat-derived peptide has a transducing activity, C45D18 was more active than Tat peptide for trafficking proteins into cells. Taking together with results from FACS analysis revealing that more than 90% of CBMN cells were positive for X-gal staining after treatment of C45D18-conjugated β-galactosidase, we propose that C45D18 translocates bioactive macromolecules directly into the nucleus.
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Keywords: HIV-1; Vpr; Nuclear trafficking; Protein transduction domain; Resting cells

Vpr, one of six auxiliary genes of human immunodeficiency virus type 1 (HIV) [1,2] encodes a virion-associated protein [3–5], and has been proposed as a factor crucial for HIV-1 infection in resting macrophages [6]. Several lines of evidence indicate that Vpr is involved in translocation of preintegration complex from cytoplasm to nucleus [6,7]. Vpr is a small protein composed of 96 amino acids (aa), but has several functional domains of three α-helix regions (17–29, 36–47, and 53–78, respectively), a leucine-rich region from 60 to 80 aa, and C-terminal arginine-rich region [7]. It has been noted that Vpr has two separable parts responsible for nuclear translocation [8]. On the other hand, we previously reported that Vpr induces genomic instability by causing chromosome breaks and aneuploidy [9,10]. Our experiments also revealed that the C-terminal region of Vpr is important for cell-cycle arrest, and Vpr mutant lacking C-terminal 18 aa was negative for inducing cell-cycle abnormality at the G2/M phase.

As a particularly interesting property, Vpr functions like a transacting factor, and latently infected cells restart viral production, when Vpr is extracellularly added to cells [11,12]. In addition to such an activity, Vpr can enter cells when it is added to culture medium [7,13]. Consistently, a synthetic full-length peptide of Vpr or C-half of Vpr was used for efficient transduction of plasmid DNA [14]. On the other hand, Sherman et al. [15] recently reported that Vpr could transport exogenous proteins into cells. A fusion protein of Vpr with β-galactosidase (β-gal) was also shown to enter cells.

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Such a transduction activity of Vpr is energy independent and does not require a cellular receptor [15]. As one of possibly related mechanisms of transducing activity, Vpr forms a channel in cellular membranes [16,17], and the amino-terminal region of 40 aa of Vpr with α-helix structures is responsible for the ion channel formation [17].

Proteins, such as antennapedia of *Drosophila* (ANTP) [18], VP22 of herpes simplex [19], and Tat of HIV-1 [20], are known to possess protein transduction domains (PTD). PTD enables proteins to cross biological membranes and helps them to enter the cytoplasm. It has been also reported that a variety of proteins, when expressed as chimetric proteins with the peptide, enter target cells. PTD has an arginine-rich region, and it was expected that the C-terminal region of Vpr, which contains an arginine-rich stretch, functioned as PTD. It was, however, concluded that the C-terminal half of Vpr did not show any activities as PTD [15], and the region of Vpr responsible for transducing exogenous protein remained to be clarified.

In the present study, we identified a sequence corresponding to the third α-helix domain (C45D18) as PTD. Interestingly, C45D18 entered cells even without cellular growth, and C45D18-conjugated green fluorescent protein (GFP) was quickly transferred to the nucleus. Transduction of protein conjugated with C45D18 was more efficient than that conjugated with Tat-derived peptide. Based on results that C45D18-conjugated proteins were efficiently transduced into cord blood mononuclear (CBMN) cells as well as resting adherent cells, we propose that C45D18 functions as a novel vehicle that facilitates nuclear trafficking of molecules into target cells.

Materials and methods

Cell culture and chemicals. HT1080 and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) (Sigma, SI). Cord blood was kindly provided by volunteers who gave informed consent. CBMN cells were prepared by centrifugation, according to the manufacturer's protocol (Nycomed Pharma AS, Norway). Briefly, cord blood was diluted with the same amount of phosphate buffered saline (PBS) and applied on the Lymphoprep solution. After centrifugation for 20 min at 800*g*, cells at the interphase were collected, washed once with PBS, and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS. Jurkat cells and HL-60 cells were cultured in IMDM with 10% FCS. To prepare resting cells, HT1080 cells were cultured for 4 days in FCS-free medium. Cell growth of HL-60 cells was also arrested with 1 μg/ml aphidicolin (APC) (Sigma, SI). As a control, dimethyl sulfoxide (DMSO), used as a solvent of APC, was treated.

Peptide synthesis and detection of incorporated peptide. Various types of peptides derived from Vpr (see Fig. 1A) and Tat (GYGRKKRR QRRRGGC, amino acids described as single letters) were chemically synthesized (Wako, Tokyo). Biotin was added at the amino terminal end of each peptide. After treatment of peptides, cells were washed once with PBS and then fixed with 100% ice-cold methanol. To exclude signals associated with cellular membranes, cells were treated for 10 min with 0.2% Triton X-100 in PBS [21]. Cells were then reacted for 1 h with streptavidin (SA)-conjugated FITC (SA-FITC) and washed several times in PBS with 0.05% Tween 20. To detect the interaction of the peptide and plasmid DNA, different doses of the peptide (1–30 μg) were mixed with 0.2 μg plasmid DNA. A reporter plasmid, pCMV/luciferase, was kindly provided by Dr. Shimada (Nihon Medical School). Luciferase activity was assayed, as described [21].

Expression of recombinant green fluorescent protein and conjugation with peptides. A recombinant protein of green fluorescent protein (GFP) tagged with $(His)_6$ was expressed by a baculovirus system with pFASTBAC and purified with proband region (Invitrogen, Carlsbad, CA). Molecular weights of GFP and β-galactosidase (β-gal) (Wako, MI) were about 35 and 465 kDa, respectively. These proteins were chemically conjugated with Vpr-derived peptides (IBL, Fujioka, Japan). Briefly, about 300 μg protein was suspended in 10 mM phosphate buffer (pH 7.0) and added with 0.1 mM *N*-[e-maleimidocaproyloxiy]succinimide ester (DOJINDO Lab. Kumamoto, Japan). After 30 min at room temperature, each Vpr-derived peptide was added and further incubated for 3 h at room temperature. Conjugated molecules were then dialyzed against PBS overnight.

To test protein transduction, cells were incubated with conjugated proteins overnight and incorporated GFP was detected by an antibody. To demonstrate β-gal activity, X-gal staining was carried out according to the method described [22].

Fluorescent activated cell sorter (FACS) analysis. Incorporation of peptides was analyzed by detecting SA-FITC bound to the peptides. For cell-cycle analysis, cells were treated for 1 h with 10 μM bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO). After fixation in 70% ice-cold ethanol, cells were treated with FITC-conjugated anti-BrdU antibody (Beckton–Dickinson, San Jose, CA) and then stained with 5 μM propidium iodide (Sigma). To study the effect of Vpr on cell-cycle, cells after treatment of peptides were stained with 50 μg/ml propidium iodide and subjected to FACS analysis. For FACS analysis of β-galactosidase activity, a FluoReporter lacZ Flow Cytometry Kit (Molecular Probes, Eugene, OR) was used. Briefly, 5×10^5/100 μl of CBMN cells was mixed with 1 mM fluorescein di-β-D-galactopyranoside for 1 min and was added to 1.8 ml of ice-cold PBS containing 1.5 μM propidium iodide. FACS analysis was carried out by Cellquest (Beckton–Dickinson, San Jose, CA).

Results

Identification of Vpr-derived oligopeptide with transducing activity

The carboxyl-half of Vpr has been shown to transduce plasmid DNA into cultured cells [14]. On the other hand, we previously reported that Vpr induced cell-cycle abnormality at the G2/M phase, but Vpr mutant that lacked C-terminal 18 amino acids was negative for the cell-cycle abnormality [9]. Based on these observations, we tested whether C-terminal 45 aa of Vpr without the extreme C-terminal 18 aa (C45D18, Fig. 1A) had a trafficking activity. A biotin-conjugated 27-mer peptide (52–78 aa) was synthesized, and 10 μg/ml of the peptide was added into the medium of cultured cells. On the next day, an incorporated peptide was detected with SA-FITC. As shown in Fig. 1B, C45D18 was clearly detected in the peptide-treated cells (Fig. 1B, middle

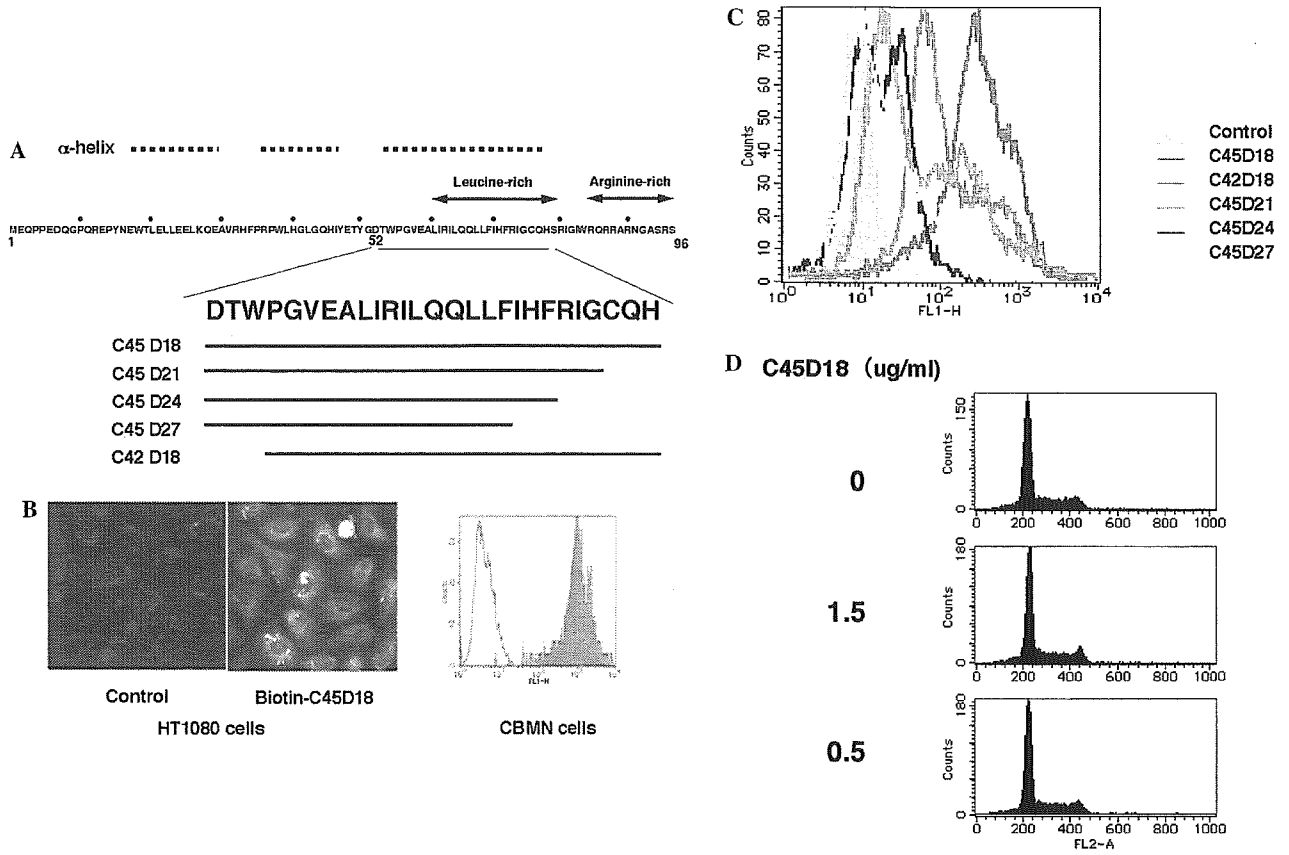

Fig. 1. Identification of Vpr-derived peptide that is incorporated into cells. (A) Amino acid sequence of Vpr used in the present study. (B) Incorporation of C45D18 into cells. Results of HT1080 cells (left panels) and CBMN cells (right panel) are shown. Note that almost all of cells are positive for the incorporated peptide (shown by yellow and red in left and right panels, respectively). (C) Transducing activity of synthetic peptides. Several biotin-conjugated peptides were synthesized and added into the culture medium of CBMN cells. On the next day, the incorporated peptides were detected with SA-FITC. Amino acid sequence of each peptide is shown in (A). (D) Effects of C45D18 on cell-cycle. Cells were treated with C45D18 for 2 days and then subjected to cell-cycle analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

panel). We observed that C45D18 was also efficiently incorporated into CBMN cells (Fig. 1B, right panel shown by red). FACS analysis revealed that almost 100% of cells were positive for the incorporated peptide after overnight treatment.

To identify the minimal region required for such trafficking activity, several biotinylated peptides were synthesized (Fig. 1A), and we tested whether they were incorporated into CBMN cells, (Fig. 1C). Three peptides of C45D21 (52–75 aa), C45D24 (52–72 aa), and C45D27 (52–69 aa) were less efficiently incorporated to CBMN cells than C45D18 (orange, purple, and yellow peaks, respectively). When amino-terminal three amino acids were deleted from C45D18 (C42D18), its trafficking activity was greatly reduced (blue in Fig. 1C).

It has been reported that Vpr induces cell-cycle abnormality at G2/M phase, and we studied whether C45D18 has an activity on cell-cycle. As shown in Fig. 1D, FACS analysis revealed that cell-cycle was not changed after treatment for 2 days. These data imply that C45D18 is an appropriate sequence for further characterization of the potentiality for transducing activity.

Trafficking macromolecules

We next studied whether C45D18 could transduce plasmid DNA. Consistent with a previous report on the full-length peptide of Vpr [14], C45D18 interacted with plasmid DNA. Unfortunately, however, we could not obtain a favorable amount of exogenous gene expression in cells transfected with the complex (data not shown). To evaluate the activity of C45D18 to transport macromolecules into cells, we studied whether C45D18, when attached to a recombinant protein, entered cells. For this purpose, C45D18 was conjugated at various molar ratios with a purified recombinant protein of GFP and added into the culture medium. On the next day, incorporated proteins were detected. As shown in Fig. 2A, cells treated with C45D18-conjugated GFP were positive for incorporation, although the protein was not detected at all in cells treated with GFP by itself (Fig. 2A, left panel). In the present study, cells were treated with 0.2% Triton X-100 before treatment with SA-FITC. Since this procedure abolished signals associated with cellular membranes [21], our positive ob-

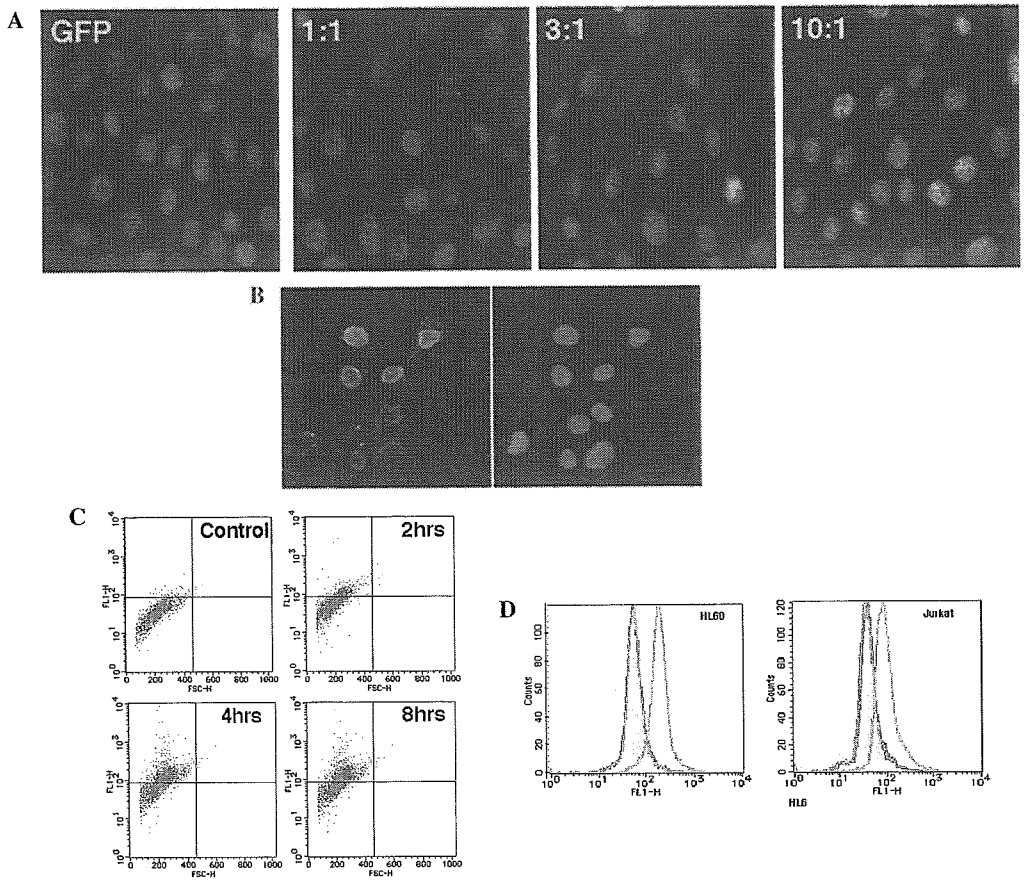

Fig. 2. Trafficking macromolecule by C45D18. (A) Incorporation of C45D18-conjugated GFP into HT1080 cells. GFP conjugated with different doses of C45D18 (3 μg/ml) was added to cells, and the incorporated GFP was detected by immunostaining with an antibody to GFP. The molar ratio of C45D18 to GFP was 1:1, 3:1, and 10:1. As a control just GFP (3 μg/ml) was added into the medium (left panel). (B) Nuclear localization of incorporated C45D18-conjugated GFP. GFP and nuclear DNA were stained with the antibody to GFP and Hoechst 33258. Incorporated GFP was detected by laser-scanning microscopy. Signals of GFP (left panel) and DNA (right panel) on the same field are shown by red and blue, respectively. (C) Time course of the incorporation of CV45D18-conjugated GFP. HL-60 cells were treated with C45D18-conjugated GFP (a molar ration of C45D18: GFP = 10:1) for 2 (upper right panel), 4 (lower left panel), and 8 h (lower right panel). As a control, cells were incubated with the conjugated GFP for 8 h. (D) Efficient trafficking by C45D18 compared to Tat-derived peptide. A chemically synthesized Tat-derived peptide (see Materials and methods) was conjugated to GFP according to the completely same procedures of C45D18, and added into culture medium of HL-60 (left panel) and Jurkat cells (right panel). Incorporated GFP, C45D18-GFP, and Tat-GFP were shown by red, orange, and blue, respectively. Note that C45D18-conjugated GFP was more efficiently incorporated than Tat-GFP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

servations indicate that the C45D18-conjugated protein was actually incorporated into cells. The amount of incorporated proteins increased according to doses of C45D18 conjugated to the protein (Fig. 2A). As a further interesting observation, incorporated GFP was detected in the regions corresponding to the nuclei of treated cells. Laser-scanning microscopy clearly detected that the incorporated GFP was present in the nucleus (Fig. 2B, see also Fig. 3C), implying that C45D18 can be used for nuclear trafficking of macromolecules.

Characterization of C45D18-dependent trafficking of macromolecules

We characterized the C45D18-dependent incorporation of GFP. To accurately measure the population with incorporated GFP, HL-60 cells were treated with the C45D18-GFP (molar ratio = 10:1) and subjected to FACS analysis. First, the dose–response of the incorporation was studied. When cells were incubated with 6, 3, and 1.5 μg/ml of the conjugated protein, 70%, 50%, and 30% of cells were positive for the incorporated GFP, respectively (data not shown). The time-course analysis was next carried out using 3 μg/ml of the conjugated protein. The incorporation of peptide-conjugated GFP was observed within 2 h after treatment (Fig. 2B). About 30% of cells were positive for the protein (Fig. 2C, upper right panel). Then, about 50% of cells were positive for the incorporated GFP in 4 or 8 h (Fig. 2B, lower panels), indicating that most of the C45D18-dependent incorporation of conjugated protein was complete within several hours.

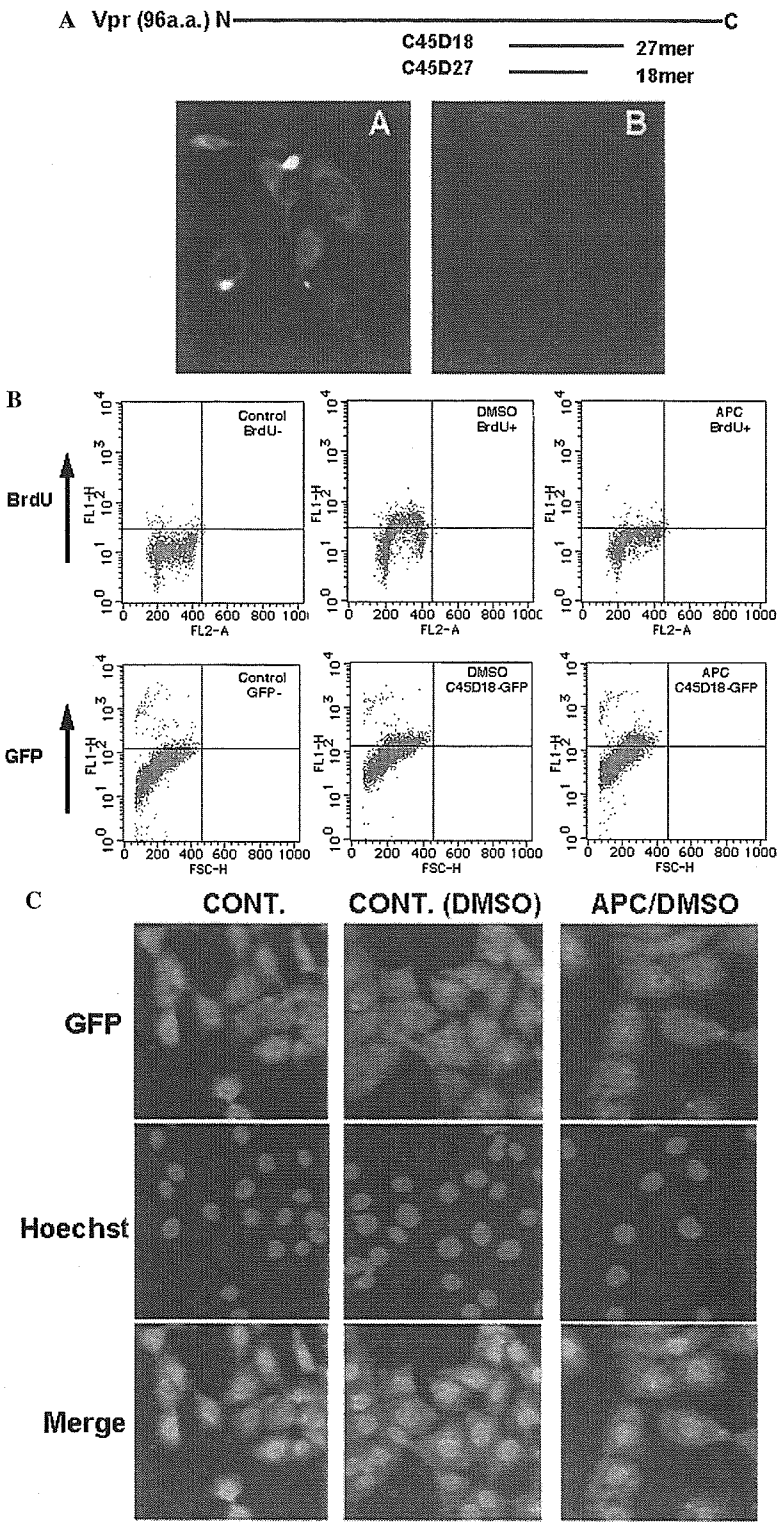

Fig. 3. Characterization of C45D18-dependent incorporation of GFP. (A) Incorporation of C45D18 into resting cells. HT1080 cells were arrested by serum-starvation for 4 days and then C45D18 or C47D27 was treated for about 12 h. Cell cycle arrest was confirmed by BrdU incorporation, followed by FACS analysis (data not shown). The incorporated peptides were detected with SA-FITC. Note that only C45D18 was incorporated into cells. (B) Incorporation of C45D18-conjugated GFP into resting cells. HL-60 cells were treated for 14 h with 1 μg/ml aphidicolin (APC), and then 3 μg/ml C45D18-conjugated GFP was treated for 5 h. Upper and lower panels show results of cell cycle analysis and incorporated GFP, respectively. As a control, cells were treated with DMSO, used as a solvent of APC (middle panels). After APC treatment, cells did not incorporate BrdU (upper right panel), but the incorporated GFP was detected in these cells (lower right panel). (C) Incorporation of C45D18-GFP into nucleus even under cell cycle arrest. HT1080 cells were treated for 14 h with 1 μg/ml APC and incubated with the conjugated GFP. The incorporated GFP was analyzed by laser-scanning microscopy. Results of C45D18-GFP added to control cells (left panels), DMSO-treated cells (middle panels), and APC-treated cells (right panels) are shown. Positive signals of the incorporated GFP (upper panels), DNA stained by Hoechst 33258 (middle panels), and merged images (lower panels) are shown.

It has been proposed that Tat, another accessory gene product of HIV-1, has a sequence of 9-mer aa with trafficking activity [20]. To compare the activity of C45D18 and Tat peptides, GFP was conjugated with each peptide at the same molar ratio (10:1) by the same procedure with C45D18. Then each protein was added to the culture media of two human cell lines, HL-60 and Jurkat cells. As shown in Fig. 2D, GFP conjugated with Tat peptide was not efficiently incorporated (blue), compared to the C45D18 (orange). In the present work, we conjugated peptides with protein through maleimide molecules and then directly added them to the culture medium without denaturing conjugated proteins. Since it is reported that Tat activity to transduce proteins is observed only after denaturing proteins [23], it may be possible that Tat activity of protein transduction is possibly detected after denaturing molecules.

It has been reported that Vpr has an activity to form channels in the cytoplasmic membrane [16,17], by which Vpr might be incorporated into cells. To exclude the possibility that trafficking of exogenous proteins is due to passive incorporation through membrane channels formed by Vpr-derived peptide, we added C45D18 and unconjugated GFP simultaneously, and then evaluated whether GFP was detected in the treated cells. No incorporated signals were observed (data not shown), indicating that C45D18 was active for transducing protein, only when it was conjugated with macromolecule.

Trafficking molecules into resting cells

It has been proposed that Vpr is responsible for infection of HIV to resting macrophages [6]. To know whether C45D18 could be incorporated into resting cells, HT1080 cells were first cultured for 4 days in FCS-free medium and then incubated with the peptide. FACS analysis on BrdU-positive cells clearly indicated that cells were not S-phase (data not shown). When C45D18 was treated with these cells, the peptide was again efficiently incorporated (Fig. 3A-A). By contrast, C45D28, a smaller peptide, was not incorporated at all (Figs. 3A and B), indicating that the incorporation of C45D18 was not due to the passive transport of small molecules into

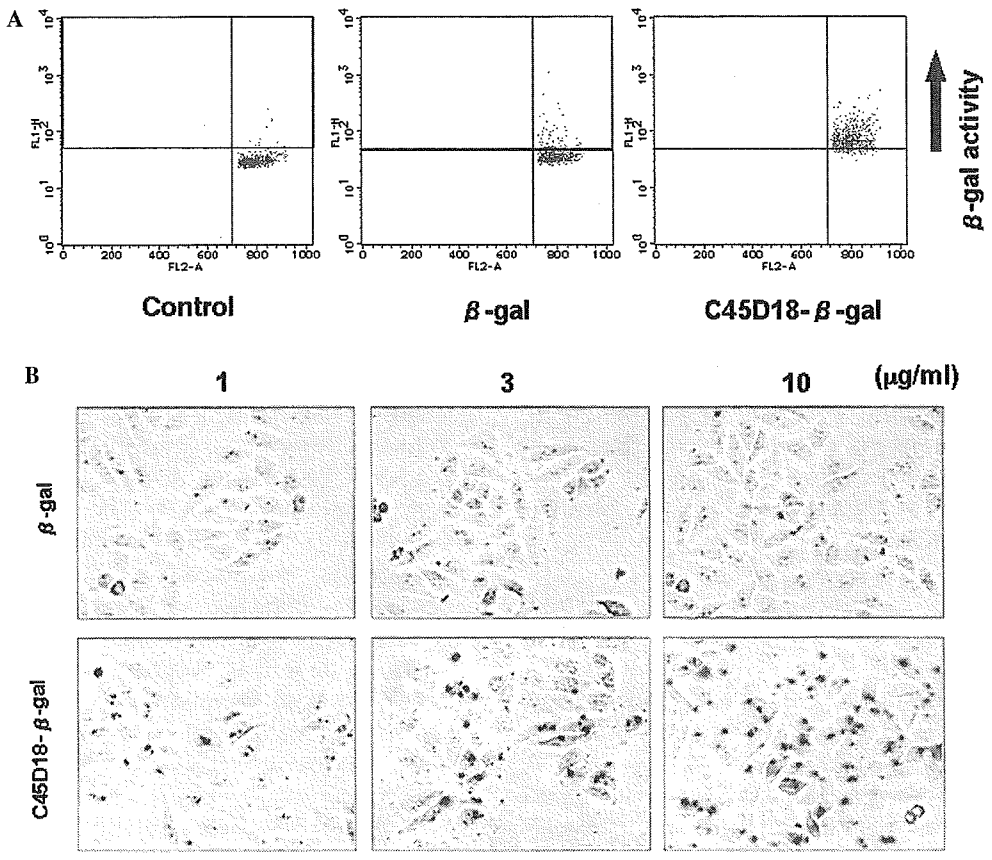

Fig. 4. Incorporation of bioactive molecule of C45D18. (A) Nuclear trafficking of active β-gal into adherent cells. HT1080 cells were treated overnight with control β-gal (upper panels) or C45D18-conjugated β-gal (lower panels). Then X-gal staining was carried out. Doses of treated proteins were 1 (left panels), 3 (middle panels), and 10 (right panels) μg/ml, respectively. β-Gal activity is indicated as black spots. Note that signals of β-gal activity are observed in regions corresponding to nucleus. (B) Incorporation of β-gal into CBMN cells by C45D18. CBMN cells were treated with C45D18-conjugated β-gal, and the activity of β-gal was detected by FACS analysis with a FluoReporter lacZ Flow Cytometry kit. Results of control (left panel), β-gal (middle panel), and β-gal conjugated with C45D18 (right panel) are shown.

cells. We next studied whether C45D18-conjugated protein was also incorporated into resting cells. HL-60 cells were first treated for 14 h with 1 μg/ml APC and then incubated for another 5 h with 3 μg/ml C45D18-conjugated GFP. We confirmed that cell cycle was completely arrested, as judged by incorporation of BrdU (Fig. 3B, upper right panel). Even under such condition, more than 50% of cells were positive for incorporated GFP (Fig. 3B, lower right panel). The same experiment was carried out on HT1080 cells and consistent results were obtained (Fig. 3C). Incorporation of C45D18-conjugated GFP into APC-treated cells was observed at almost the same level with control (Fig. 3D, right panels). Interestingly, the incorporated GFP was again detected in nuclear regions, judged by laser-scanning microscopy (Fig. 3C, middle and right panels). These data indicate that the nuclear trafficking of protein by C45D18 was not dependent on cellular growth.

Nuclear trafficking of bioactive macromolecules

To know whether C45D18 could transport a bioactive macromolecule, β-gal with a molecular weight of 465 kDa was conjugated with C45D18 and then added to cells. To show bioactivity, X-gal staining was carried out on the next day. As shown in Fig. 4A, β-gal activity was clearly detected in HT1080 cells treated with conjugated β-gal. The numbers of cells positive for of X-gal staining increased in a dose-dependent manner of treated β-gal (Fig. 4A). We also observed that β-gal activity was present in regions corresponding to nucleus.

C45D18-dependent incorporation of β-gal activity was also demonstrated by FACS analysis on CBMN cells that were treated with the protein (see Materials and methods). As shown in Fig. 4B, more than 90% of treated cells were positive for β-gal activity (Fig. 4B, right panel). By contrast, treatment of β-gal alone did not increase the number of cells positive for the activity (Fig. 4B, middle panel). These data indicate that the trafficking property by C45D18 can transduce bioactive molecule at high efficiency.

Discussion

In the present study, we identified a sequence encompassing 52–78 aa of Vpr (C45D18) as a novel PTD. To confirm the reproducibility of our observations, we synthesized C45D18 more than three times and examined the activity of the peptide conjugated with proteins. Independent experiments revealed that C45D18 or its conjugated proteins, when added to culture medium of cells, were efficiently incorporated into the nuclear region.

For nuclear trafficking of proteins into cells from outside, there are at least two steps where C45D18 should function. One is that C45D18 enables conjugated proteins to cross biomembranes, and another step is that C45D18 translocates the incorporated protein to the nucleus. Although the precise mechanism remains to be clarified, it has been well proposed that Vpr enters cells [7,13], when added to the culture medium. As a possible explanation of this phenomenon, it has been proposed that Vpr forms ion channels in cellular membranes [16,17]. The region responsible for channel formation has been recognized in amino-terminal 40 aa [17]. Crossing cellular membranes by the C45D18 would not, however, be due to the ion channel formation by a proposed region, since C45D18 is located in the C-terminal half of Vpr. How the conjugated protein enters cells remains to be clarified.

As one of the most important functions of Vpr, it is involved in the nuclear trafficking of a pre-integration complex of HIV-1 (PIC) [6,24], which explains an intriguing activity of HIV-1 to infect resting macrophages [25]. The mechanism of nuclear trafficking activity of Vpr has been extensively investigated, and it is well proposed that Vpr is a nucleopheric protein [6,24,26–28]. Interestingly, however, it does not have a classical nuclear localization signal. Although there are some controversial reports [8], it has been proposed that Vpr binds karyopherin α [6,24] and translocates PIC to the nucleus. Furthermore, Vpr has been shown to interact with members of nuclear pore complex (NPC) proteins such as Nsp1p [27] and nucleoporin hCG1 [28]. Functional analysis using chimeric proteins of Vpr and β-gal has indicated that two parts of Vpr promote nuclear trafficking of β-gal. It was reported that 71–96 aa of Vpr is still active in nuclear localization. Since C45D18 has a region of 53–78 aa, it might be possible that an overlapped region of 71–78 aa has an affinity to NPC proteins, responsible for nuclear trafficking. To know whether the overlapped region 71–78 aa of Vpr (peptide-8) functions for nuclear trafficking of C45D18, we added peptide-8 into culture medium of cells and compared with the properties of C45D18. Although we observed that C45D18 was incorporated into the nuclear region, but peptide-8 was scarcely translocated to nucleus (data not shown), implying that the region of 71–78 aa of Vpr is not enough for nuclear translocation of C45D18.

As an important observation, C45D18 could transduce exogenous molecules into resting cells. Although retrovirus gene transfer is frequently utilized in clinical fields, exogenous genes cannot be transduced in resting cells by the system. To circumvent this problem, modified lentiviral vectors are developed for transducing genes into resting cells [29]. On the other hand, recent observations reveal that a retroviral system occasionally results in fatal side effects [30], implying that a non-viral gene transfer system would be more reliable in future clinical use. In most of gene transfer systems, however, the expression of exogenous genes depends on break

down of nuclear membranes. To obtain an efficient gene expression in resting cells, it is crucial to develop a system by which exogenous genes are directly transferred into nucleus. Since C45D18 can efficiently translocate into the nucleus, it is tempting to speculate that the frequency of gene expression by non-viral gene transfer systems may be improved by the combination with C45D18.

It has been reported that several peptides derived from different sources—such as ANTP, herpes simplex VP22, and nine amino acids of Tat peptide-possess protein transduction activity. When compared to the activity of Tat, C45D18 has more potent activity in transporting molecules. It has been well proposed that the transduction activity of Tat requires protein denaturing [23]. Our present work reveals that C45D18 is more versatile than Tat peptide, since C45D18-conjugated molecules can be directly utilized for nuclear trafficking without any subsequent procedures. Additionally, C45D18 did not induce any cell-cycle abnormality or apoptosis (Fig. 1D), implying that it can be used without serious side effects.

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