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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.

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

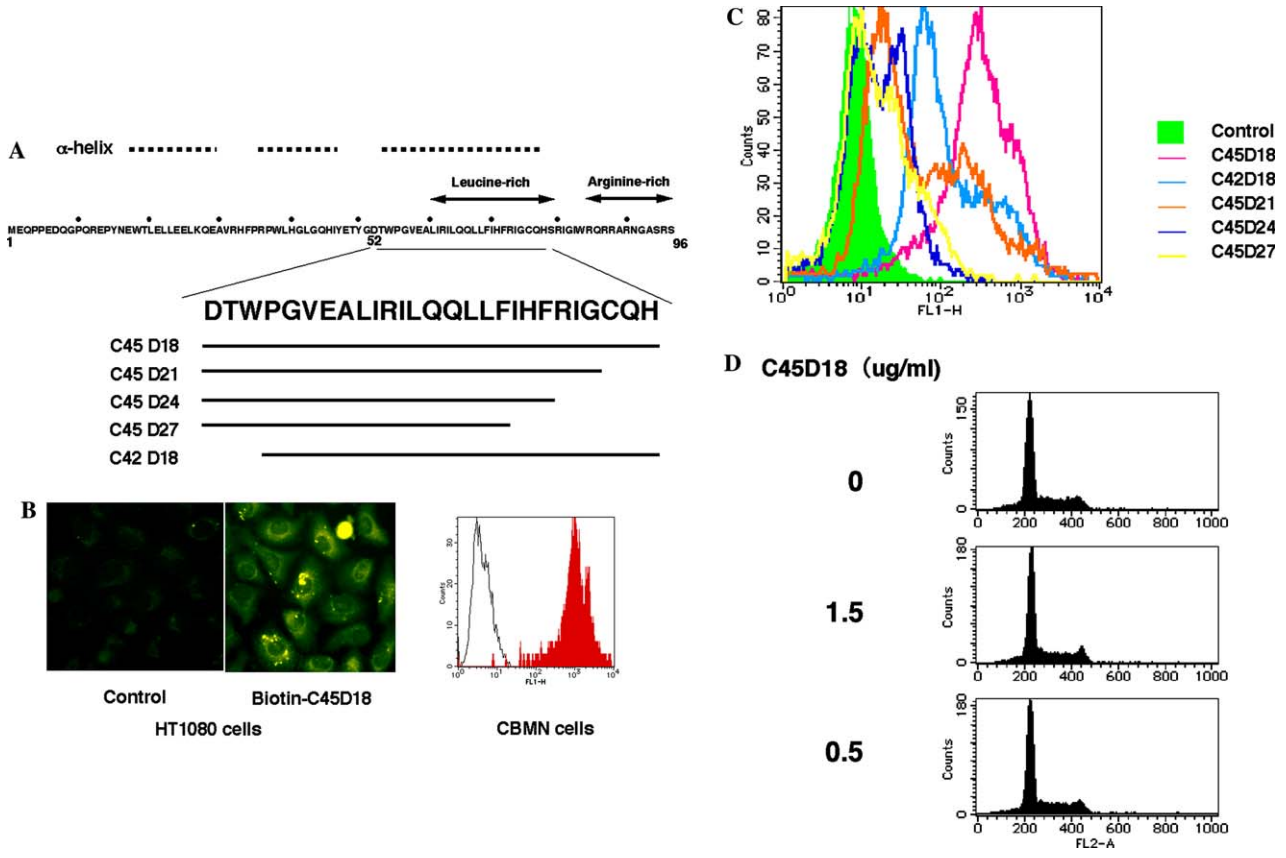

Fig. 1. Identification of Vpr-derived peptide that is incorporated into cells. (A) Amino acid sequence of Vpr used in the present study. (B) Incorporation of C45D18 into cells. Results of HT1080 cells (left panels) and CBMN cells (right panel) are shown. Note that almost all of cells are positive for 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-

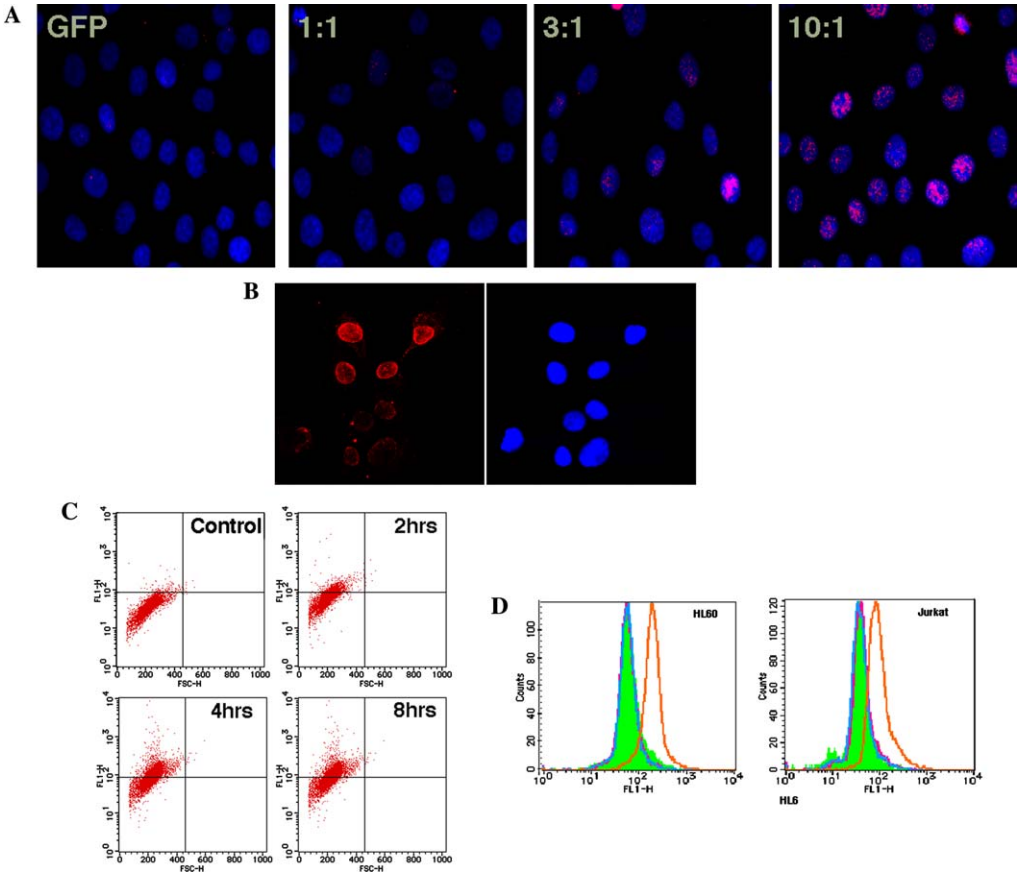

Fig. 2. Trafficking macromolecule by C45D18. (A) Incorporation of C45D18-conjugated GFP into HT1080 cells. GFP conjugated with different doses of C45D18 (3 μg/ml) was added to cells, and the incorporated GFP was detected by immunostaining with an antibody to GFP. The molar ratio of C45D18 to GFP was 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.

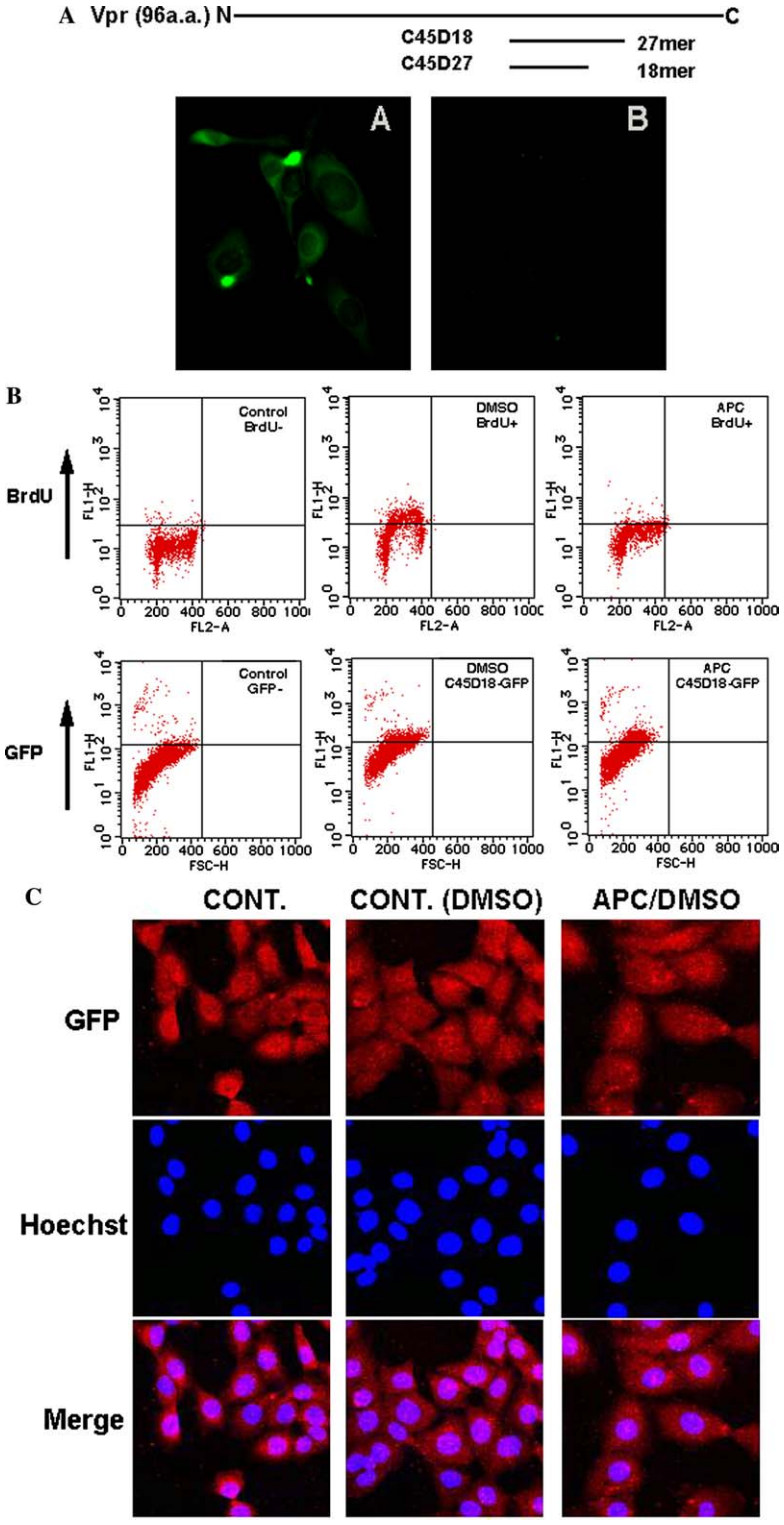

Fig. 3. Characterization of C45D18-dependent incorporation of GFP. (A) Incorporation of C45D18 into resting cells. HT1080 cells were arrested by serum-starvation for 4 days and then C45D18 or C47D27 was treated for about 12 h. Cell cycle arrest was confirmed by BrdU incorporation, followed by FACS analysis (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

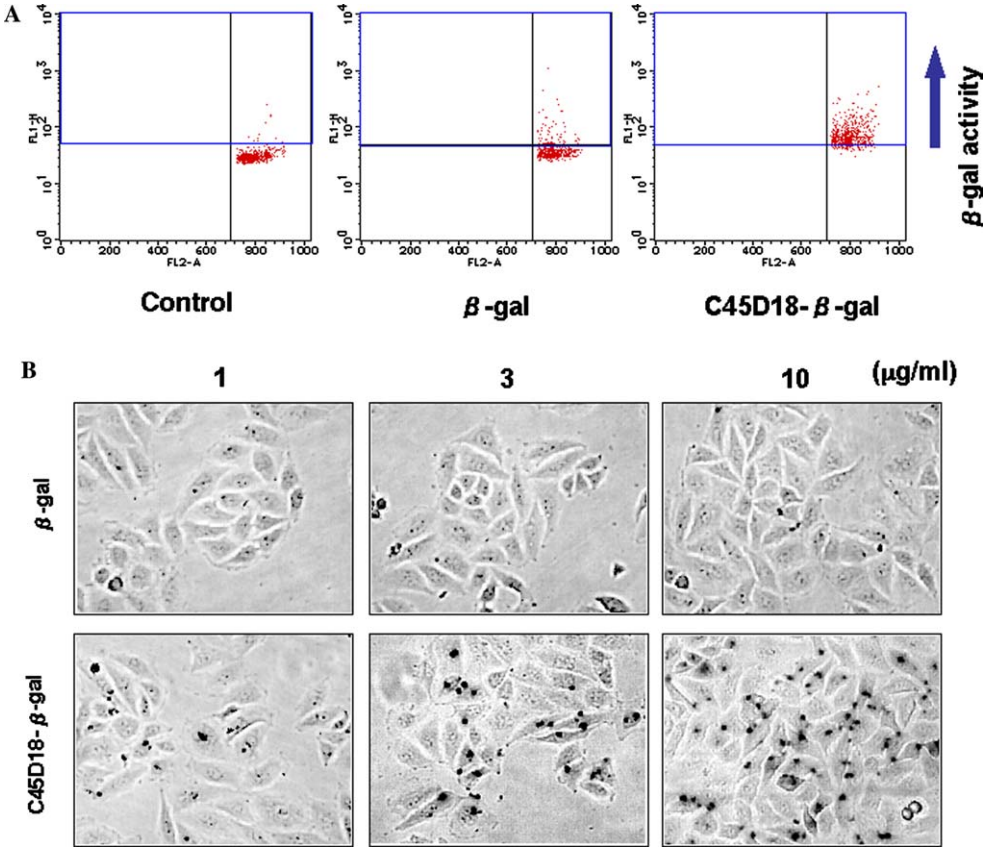

Fig. 4. Incorporation of bioactive molecule of C45D18. (A) Nuclear trafficking of active β-gal into adherent cells. HT1080 cells were treated overnight with control β-gal (upper panels) or C45D18-conjugated β-gal (lower panels). Then X-gal staining was carried out. Doses of treated proteins were 1 (left 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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