Identification of Adenovirus-Derived Cell-Penetrating Peptide

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Cell-penetrating peptides (CPPs) have been highly anticipated as an efficient delivery system due to their ability to cross biological membranes and transport various cargoes into cells. In the present study, we have identified adenovirus-derived CPPs using various capsid-mutant adenovirus (Ad) vectors. First, we examined the endocytosis-inducing ability of these vectors. A fiber-shaft substituted Ad vector, Ad type 5 vector with the fiber shaft domain replaced by that derived from Ad type 35, induced the highest fluorescein isothiocyanate (FITC)-dextran uptake into a human liver cell line, HepG2 cells. In contrast, the FITC-dextran uptake in HepG2 cells was not significantly different between coxsackievirus and adenovirus receptor (CAR)-binding-ablated Ad vector, integrin-binding-ablated Ad vector or conventional Ad vector. Next, we produced a recombinant Ad type 35 shaft protein using the Escherichia coli recombinant system. The recombinant Ad type 35 shaft protein retained the ability for FITC-dextran uptake and efficient gene delivery by plasmid transfection reagent. Furthermore, we identified 26 C-terminal amino acids in the Ad type 35 shaft protein as the cell membrane binding domain. The 26 amino-acid peptides also have the potential to be internalized into cultured cells. The internalization ability of the peptide was dependent on degree and was inhibited by an actin polymerization inhibitor (Latrunculin B) and by a lipid raft formation inhibitor (methyl-

Key words  cell-penetrating peptide; adenovirus; endocytosis

Recombinant adenovirus (Ad) vectors are useful for gene transfer to cultured cells and animal tissues both in vitro and in vivo. The initial step of type 5 Ad infection involves at least two sequential steps. The first is the attachment of the virus to the cell surface by binding of the knob domain of the fiber to coxsackievirus and adenovirus receptor (CAR).1,2) Following attachment, interaction between the RGD motif of penton bases with the secondary host-cell receptors, αvβ3- and αvβ5-integrin, facilitates the internalization of the vector via receptor-mediated endocytosis.3,4) Furthermore, the interaction between the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of type 5 Ad with heparan sulfate proteoglycans (HSPG) and the length of the fiber shaft are involved in accumulation in the mouse and cynomolgus monkey livers of systemically administered Ad vectors.5,6) On the other hand, type 35 Ad vector, subgroup B Ad, binds to human CD46 as an attachment receptor. Following attachment, the RGD motif of penton bases interacts with the host-cell αv-integrin, similar to subgroup C type 5 Ad vector.8) Internized Ad reaches the endosomal pathway and avoids lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus.9) Efficient gene transfer of Ad vectors is achieved by these cell entry mechanisms. When compared with one copy, the gene expression efficiency of Ad vector is known to be ten thousand times higher than gene transfer by a complex cationic polymer and plasmid; part of the difference is due to intracellular trafficking, including plasma membrane penetration.10,11) Uptake of type 5 Ad vector is mediated via clathrin-mediated endocytosis by interaction between the RGD motif and integrin. In contrast, species B Ad viruses, such as type 3 Ad and type 35 Ad, are internalized by macropinocytosis.12,13) Furthermore, species C Ad viruses, such as types 2 and 5 Ad, induce macropinocytosis and leakage of macropinosomal contents into the cytosol, as shown by codelivery assays.14) This uptake pathway could be delivery of the cointernalized dextran into the cytosol, but at least for type 2 Ad macropinocytosis is not the infectious entry pathway.14) This macromolecule uptake pathway into the cells by Ad capsid protein has become a powerful tool for efficient intracellular delivery of molecules such as therapeutic nucleic acid and proteins. The identification of functional domains is required before the pro-

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tein can be used as a therapeutic drug delivery system.

Several viral functional proteins are used in basic studies of drug targeting as their unique function. One category, cell-penetrating peptides (CPPs), is capable of transporting macromolecules across cell membranes, enabling their delivery to the cytoplasm, cell organelles and various tissues for execution of their different functions.\(^{15}\) CPPs are generally short peptides consisting of less than 30 amino acids in which a polypeptide motif derived from natural proteins with penetrating functions can be distinguished.\(^ {15}\) Many CPPs have been found and are used in biomedical research and drug delivery systems. In previous studies, various CPPs had different capacities for internalizing through the plasma membrane, with some being capable of penetrating bio-membranes, such as the Blood–Brain Barrier, gastric membrane and skin.\(^ {15}\) For the optimization of tissue membrane permeability by CPPs, it becomes necessary to use different CPPs for each tissue of the target. One of the major origins of CPPs is viral proteins, such as the Tat peptide from human immunodeficiency virus (HIV).\(^ {16}\) Tat protein is a RNA-binding protein involved in transcriptional control of HIV; it is independent of the entry of HIV into cells. While elucidating the function of Tat protein, it was found to penetrate into the cell. Subsequently, its RNA-binding region (positions 48–60) was revealed to play an essential role in its intracellular penetration. These studies suggest that by investigating the unknown function of viral proteins in detail, it may be possible to discover functional proteins available for research of drug delivery systems (DDS).

In the present study, we report that type 35 Ad-derived peptides induce endocytosis in cultured cells and have the ability to cross biological membranes. This report is the first paper to identify Ad-derived CPPs.

MATERIALS AND METHODS

**Cells and Reagents** HepG2, a human liver cell line, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium containing 10% fetal bovine serum under an atmosphere of 5% CO\(_2\). K562, a chronic myelogenous leukemia cell line from a patient in blast crisis, was maintained in RPMI-1640 medium containing 10% fetal bovine serum under an atmosphere of 5% CO\(_2\). Fluorescein isothiocyanate (FITC)-dextran with a molecular weight of 40000 (FD-40) was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG), fluorescein-labeled goat anti-mouse IgG (H+L) and anti-His-tag antibody were obtained from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.), Invitrogen (Carlsbad, CA, U.S.A.) and Novagen (Madison, WI, U.S.A.), respectively. Fe R Blocking Reagent human was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Latrunculin B (LatB) was obtained from CALBIOCHEM (Darmstadt, Germany). Methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD) was obtained from Sigma-Aldrich.

**Ad Vector Preparation** We used conventional Ad vector, Ad-L2, and mutant Ad vectors, AdΔF-L2, AdΔP-L2 and AdΔS-L2 are ablated of CAR-, integrin- and HSPG-binding, respectively. AdΔS35-L2 is a fiber-structure substituted Ad vector in which the fiber shaft domain of the Ad type 5 vector is replaced by that derived from Ad type 35. Vectors were prepared as described previously and purified by CsCl\(_2\) step gradient ultracentrifugation followed by CsCl\(_2\) linear gradient ultracentrifugation.\(^ {17,19}\) The particle (VP) titer of the vectors was calculated according to the report by Maizel et al.\(^ {20}\)

**Ad-Mediated FD40 Uptake into HepG2 Cells** HepG2 cells were seeded into a 24-well plate. After 3 d, they were treated with mutant Ad vectors (1000 VP/cell) with 0.5 mg/mL FD-40 for 1.0 h. After a 1.0 h culture period, cells were washed with cold phosphate-buffered saline (PBS). Fluorescence of FD-40 in HepG2 cells was observed using an OLYMPUS confocal laser scanning microscope systems (FLUOVIEW FV-300, OLYMPUS, Tokyo, Japan). We repeated all uptake experiments and obtained similar results.

**Purification of Ad Shaft Protein** To generate histidine-tagged Ad type 5 shaft proteins (Ad5shaft), mutant Ad type 5 shaft (Ad5shaft mut) and Ad type 35 shaft (Ad35shaft), we used pAdHM4, pAdHM68 and pAdHM54 as templates, respectively.\(^ {18,19}\) The template was subjected to the polymerase chain reaction using the following oligonucleotides: *cata*GGGGAATCTTCTTIGCGC (sense primer for Ad5shaft and Ad5shaft mut; the underline indicates the NdeI site), *ggat*TTATAGCCTTTATATTITTGT (antisense primer for Ad5shaft and Ad5shaft mut; the underline indicates the BamHI site), and *cata*GGAGTTCTTTATTTAAATGT (sense primer for Ad35shaft; the underline indicates the NdeI site), *ggat*TTATAGCCTTTATTTAC (antisense primer for Ad35shaft; the underline indicates the BamHI site). The resulting fragments of Ad5shaft, Ad5shaft mut and Ad35shaft were subcloned into pGEM-T vector (Promega, Madison, WI, U.S.A.) by the TA-cloning system. The sequences of the fragments were confirmed. Ad shaft protein expression plasmids were prepared as follows: pGEM-T vector with Ad shaft was digested with NdeI/BamHI, and the resultant NdeI–BamHI fragment was inserted into the identical site of pET16b vector. Those pET16b plasmids with Ad5shaft, Ad5shaft mut and Ad35shaft were transduced into Escherichia coli BL21 (DE3), and the production of Ad5shaft, Ad5shaft mut and Ad35shaft was stimulated by the addition of isopropyl-\(\beta\)-D-thiogalactopyranoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cells were harvested, resuspended in buffer A [10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl\(_2\), 10% glycerol, 0.1 mM (\(\beta\)-amidophenyl)methanesulfonyl fluoride hydrochloride, and 1 mM \(\beta\)-mercaptoethanol], and then lysed by sonication. The lysates were applied to a Ni-column, followed by transfer to a nitrocellulose membrane. The protein samples is given above. Total protein (1.0 µg) in sample buffer with 4% \(\beta\)-mercaptoethanol was separated in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue (CBB) staining.

**CBB Stain and Western Blotting** The preparation of the protein samples is given above. Total protein (1.0 µg) in sample buffer with 4% \(\beta\)-mercaptoethanol was separated in a sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis gel, followed by transfer to a nitrocellulose membrane. After blocking in 1% skimmed milk, the filters were incubat-
ed with an anti-His-tag mouse antibody (1:2500), followed by incubation in the presence of horseradish peroxidase-labeled anti-mouse IgG goat antibody (1:10000). The filters were developed using chemiluminescence (ECL Western blotting detection system), and signals were read using an LAS-1000 (FUJIFILM Co., Tokyo, Japan).

**Recombinant Adshaf t Protein Mediated FD40 Uptake into HepG2 Cells** HepG2 cells were seeded into a 24-well plate. After 3d, they were treated with recombinant Adshaft protein (5.0 µg/mL) with 1.0 mg/mL FD-40 for 1.0h. After a 1.0h culture period, cells were washed with cold PBS. Fluorescence of FD-40 in HepG2 cells was observed using an OLYMPUS confocal laser scanning microscope systems (FLUOVIEW FV-300). We repeated all uptake experiments and obtained similar results.

**Immunofluorescence Microscopy** HepG2 cells were seeded into a 24-well plate. After 3d, they were treated with recombinant Adshaft protein (50 µg/mL) with 0.1 mg/mL FD-40 at 4°C for 1h. After a 1h culture period, cells were washed with cold PBS. After PBS wash, cells incubated at 37°C in culture medium for 15, 30min or 8h. Cells were fixed in 1% formaldehyde at 4°C for 3h and incubated in 0.1% Triton X-100 for 15 min. After rinsing with PBS, fixed cells were blocked with 5% BSA in PBS for 45 min and incubated with an anti-His-tag mouse antibody (1:500) at room temperature (RT) for 1h. After washing PBS, cells were incubated with the respective secondary antibodies at RT for 1h. After washing again in PBS. Images of cells were generated using an OLYMPUS confocal laser scanning microscope system (FLUOVIEW FV-300).

**Preparation of Peptides** Peptides from the sequence of Adshaft were obtained from Gene-Script (Gene-Script Japan, Tokyo, Japan). Peptide A is from the N-terminal 45 amino acid residue of Adshaft (NH$_2$-GVLTLKLCCLP LTTGGSGLQL KVGGGLTVDL TDGTLQENIR ATAPI- COOH). Peptide B is from the C-terminal 44 amino acid residue of Adshaft (NH$_2$-TKNNHISVELS IGNGLSEQKNL KCAKLGNLFKKNNDCIK DSIN-COOH). Peptide C is from the C-terminal 26 amino acid residue of Peptide B (NH$_2$-NNKCAK LGGLKFNNGDC IKDSIN-COOH). The purity of the peptide was >90%. The peptide was dissolved in PBS buffer and stored at −80°C before use.

**Reporter Gene Assay** We used pCMVL1, which has a CMV promoter and firefly luciferase gene, for the reporter gene assay. $^{23}$ The plasmid transfection complex was produced using Effectene reagent (Qiagen, Inc., Valencia, CA, U.S.A.). HepG2 cells were seeded into a 24-well plate. After 3d, they were treated with Ad5shaft, Ad5shaft mut and Ad35shaft (1.0 µg/mL) with plasmid transfection complex for 1.5h. After cultivating for 48h, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co., Ltd., Tokyo, Japan).

**Flow Cytometry** The cells (5×10$^5$ cells) were single-cell suspended using 2 mM ethylenediaminetetraacetic acid (EDTA)/PBS. The suspended cells were treated with 1.6 mg/mL trypsin/PBS (DIFCO LABORATORIES) or 40 µIU/mL heparinase I/PBS for 15 or 30 min, respectively, at 37°C, followed by treatment with Ad35shaft (50 µg/mL) for 30 min on ice. After washing, the cells were incubated with anti-His-tag mouse monoclonal antibody. Then, the cells were incubated with FITC-labeled goat anti-mouse IgG as the second antibody and analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

The cells (5×10$^5$ cells) were single-cell suspended using 2 mM EDTA/PBS. The suspended cells were treated with 30 µg/mL Peptide A, B and C for 10 min on ice, followed by addition of 30 µg/mL Ad35shaft for 60 min on ice. After washing, the cells were incubated with anti-His-tag mouse monoclonal antibody. Then, the cells were incubated with FITC-labeled goat anti-mouse IgG as the second antibody and analyzed by flow cytometry. The mean fluorescence intensity was analyzed with Cell Quest software (Becton Dickinson).

**Statistical Analyses** Data are presented as the mean±standard deviation (S.D.). Comparisons between cell types were made using Students’ t-test and differences were considered significant at p<0.05.

## RESULTS

**Ad-Mediated FD40 Uptake into HepG2 Cells** To examine the contribution of the fiber protein to Ad vector-mediated endocytosis in vitro, we examined the FD-40 uptake in HepG2 cells using capsid-mutant Ad vectors. We used conventional Ad vector, Ad-L2, and mutant Ad vectors AdAF-L2, AdAP-L2 and AdAS-L2 are ablated of CAR, integrin, and HSPG binding, respectively. AdAS35-L2 is a fiber-shaft-substituted Ad type 5 vector in which the the fiber shaft domain is replaced with that derived from Ad type 35 (Table 1). HepG2 cells are CAR-positive and express the αvβ3 and αvβ5-integrins (data not shown). FD-40 uptake in HepG2 cells was not significantly different between the treatment with AdAF-L2 or AdAP-L2 and with Ad-L2. This result suggests that there is no correlation in the FD-40 uptake and Ad vector binding ability of CAR or αv-integrin (Fig. 1). On the other hand, AdAS-L2 and AdAS35-L2 induced a larger uptake of FD-40 in HepG2 cells than Ad-L2. Furthermore, AdAS35-L2 induced a larger uptake

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of FD-40 in HepG2 cells than AdΔS-L2. There is no binding domain for HSPG in the type 35 Ad fiber shaft such as the KKTK motif in the type 5 Ad fiber shaft. These results suggest that the binding ability of HSPG in type 5 Ad vector decreases the uptake of FD-40 in HepG2 cells, and the type 35 Ad fiber shaft indicates a potential to enhance FD-40 uptake.

Effect of Recombinant Ad Shaft Protein on Cellular Uptake

The Ad type 5 shaft, the region between amino acids 47 and 399 in the fiber protein, is responsible for the interaction with HSPG. On the other hand, the Ad type 35 shaft, the region between amino acids 44 and 132 in the fiber protein, is not known to interact with cellular molecules. To evaluate the function of the fiber shaft region of Ad types 5 or 35 on the uptake of FD-40, we prepared Ad5shaft, Ad5shaft mut and Ad35shaft, which contain a whole Ad type 5 shaft, a four amino acid mutation of Ad type 5 shaft (the KKTK motif is changed to GAGA) and a whole Ad type 35 shaft, respectively (Fig. 2A). Recombinant Ad shaft proteins were fused to ten consecutive histidine residues to facilitate purification. Recombinant Ad shaft proteins were confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue and Western blotting (Fig. 2B). To evaluate the influence of Ad shaft proteins on the uptake of FD-40, we observed changes in the intensity of fluorescence in HepG2 cells that were seeded onto glass bottom plates and treated with FD-40 and Ad shaft proteins. Compared with the non-shaft protein, the intensity of fluorescence increased with Ad5shaft, Ad5shaft mut and Ad35shaft (Fig. 3A). Increases in intensity of fluorescence were observed especially after treatment with Ad35shaft for 1 h. The Ad35shaft protein, with which the cells were treated at concentrations of 10.0, 4.0, and 1.6 µg/mL, did not affect the amount of lactate dehydrogenase (LDH) release by HepG2 cells for 24 h (data not shown). This suggested that Ad35shaft protein was not cytotoxic. To analyze the intracellular trafficking of the Ad35shaft protein, we performed immunostaining. After 15 min of co-treatment, FD-40 existed on the cell membrane surface with Ad35shaft protein (Fig. 3B). After 30 min, FD-40 and Ad35shaft protein were both partially separately present in the HepG2 cells. Some red fluorescence was observed, which is Ad35shaft protein present inside the HepG2 cells. After 8 h, FD-40 and Ad35shaft protein were both completely separately present in the HepG2 cells.

To investigate the uptake-enhancing effects of Ad shaft proteins, we performed a reporter gene assay using luciferase-producing plasmid with cationic transfection reagent. Recombinant Ad35shaft protein enhanced the luciferase production of plasmid transfection reagent (8.8-fold at 1.0 µg/mL of shaft protein). On the other hand, Ad5shaft and Ad5shaft mut enhanced similar levels of luciferase production in the control test (Fig. 3C). The recombinant Ad35shaft protein is introduced into the transfection complex also in the HepG2 cells, not only the FD-40 ones. These results suggest that the type 35 Ad fiber shaft region contains the functional domain for enhanced cellular uptake.

Cellular Binding Ability of Recombinant Ad 35 Shaft Protein

Smith et al. have shown that the KKTK motif of...
the fiber shaft of Ad type 5 interacts with HSPG and is involved in accumulation in the mouse liver of systemically administrated Ad vectors. On the other hand, the interacting molecule of the fiber shaft of Ad type 35 has not been reported. Therefore, we investigated the molecules involved in the cellular uptake of the Ad35shaft protein. First, we elucidated the Ad35shaft protein that binds to the cell surface of HepG2 and K562 cells. Binding of the Ad35shaft to HepG2 cells was completely eliminated by pretreatment with trypsin and heparinase I. Binding of the Ad35shaft to K562 cells was completely eliminated by pre-treated with trypsin, but not by pretreatment with heparinase I. The molecule of the Ad35shaft binding to both cultured cells is sensitive to trypsin. To identify the binding domain of the Ad shaft protein, we synthesized...
three peptide fragments, Peptides A, B and C are from the N-terminal 45 amino acids, C-terminal 44 amino acids and C-terminal 26 amino acids of the Ad type 35 shaft sequence, respectively (Fig. 4B). The three peptide fragments were examined for their inhibitory effect on Ad35shaft binding to HepG2 cells. Binding of Ad35shaft to HepG2 cells was inhibited by peptides B and C, but was not inhibited by peptide A (Fig. 4C). The peptides B and C inhibit the binding of Ad35shaft to HepG2 cells in a dose-dependent manner, because peptides B and C are one half and one quarter of the molecular weight of the Ad35shaft protein, respectively. These results suggest that the binding domain of the Ad shaft protein is contained in the C-terminal 26 amino acids of Ad35shaft.

Cell Penetrating Function of Ad-Derived Peptide Fragment We revealed that peptide C inhibits binding of the Ad35shaft protein to the cell surface, indicating that peptide C contains the domain binding to the cell surface. Therefore, we investigated the cellular binding and cellular uptake ability of FITC-conjugated peptide C (FITC-peptide C). First, we elucidated the binding of FITC-peptide C to the cell surface of HepG2 cells and K562 cells, as well as to Ad35shaft. Binding of FITC-peptide C to both cells was completely inhibited by

Fig. 4. Binding Ability of Recombinant Ad35shaft Protein to the Cell Surface

(A) The cells (5x10^5 cells) were single-cell suspended using 2mM EDTA/PBS. The suspended cells were treated with 1.6mg/mL trypsin or 40mIU/mL heparinase I for 15 or 30min, respectively, at 37°C, followed by treatment with recombinant Ad35shaft protein (50µg/mL) for 30min on ice. After washing, immune-staining of the cells by anti-His-tag antibody was performed as described in Materials and Methods. (B) Scheme and sequence of Ad35shaft fragments. The sequence of peptide C is underlined. (C) Inhibition of binding of Ad35shaft to HepG2 cells by Ad35shaft fragments. The suspended cells were treated with 30µg/mL peptide A, B and C for 10min on ice, followed by 30µg/mL Ad35shaft added for 60min on ice. After washing, immune-staining of the cells by anti-His-tag antibody was performed as described in Materials and Methods. All data represent the mean±S.D. of four experiments.
pretreatment with trypsin, but not by pretreatment with heparinase I (Fig. 5A). Next, we examined the penetration ability of peptide C. FITC-peptide C was detected into HepG2 cells and K562 cells at 37°C. It is suggested that peptide C has cell permeability in various cells, which are hepatocytes, blood cells and cancer cells. On the other hand, FITC-peptide C was not detected into K562 cells at 4°C (Fig. 5B). This result means that the cellular uptake of peptide C does not cause cell membrane fusion. Peptide C, with which the cells were treated at concentrations of 150, 50, and 16 µg/mL did not affect the amount of LDH release at HepG2 cells or K562 cells for 24 h (data not shown).

Therefore, we investigated the cellular uptake mechanism of peptide C using inhibitors. Lat B, which is used as an en-
docytosis inhibitor, prevents actin polymerization. Because it removes cholesterol from the cell membrane, MJ/CD can be used as a lipid raft disintegrating agent. Both inhibitors impede cellular uptake of peptide C in a concentration-dependent manner (Fig. 5C). These results mean that cellular uptake of peptide C requires the presence of actin polymerization and cell membrane lipid rafts, and is caused by endocytosis.

DISCUSSION

In this study, we report that the shaft region of Ad fiber protein induces cell endocytosis; especially the Ad35 shaft region of Ad induces the highest amount of FITC–dextran uptake in cultured cells. Recombinant Ad type 35 shaft protein is shown to facilitate FITC–dextran uptake and efficient gene delivery by plasmid transfection reagent. Furthermore, the 26 amino-acid peptides also are potentially internalized into cultured cells. These results of the present study indicate that Ad type 35-derived peptides induce endocytosis in cultured cells and have the ability to cross biological membranes. This report is the first paper to identify Ad-derived CPPs.

Type 5 Ad vector interacts between the RGD motif of penton bases and the secondary host-cell receptors, αvβ3- and αvβ5-integrin, and facilitates the internalization of the vector via receptor-mediated clathrin-dependent endocytosis. On the other hand, in Type 5 Ad-infected cells some induced endosomal vehicles without Ad particles have been detected.14) This endosomal vehicle is reported to have been induced by macropinocytosis upon a signal through αv-integrins. To examine the Ad capsid protein that is involved in macropinoctosis induction, we examined FD-40 uptake into cells using several mutant Ad vectors. An uptake experiment was used to elucidate the uptake mechanism using a model substance such as transferrin or the large molecular weight dextran, as models of clathrin-dependent endocytosis or macropinocytosis, respectively. The cellular uptake of FD-40 by Ad vectors is considered to be induced even without RGD motif of penton bases (Fig. 1). FD-40 uptake into HepG2 cells is induced not only by the Ad35 shaft region in type 5 Ad vector, but also by recombinant Ad35 shaft protein (Figs. 1, 3A). The Ad35 shaft region in type 5 Ad vector and the recombinant Ad 35 shaft protein are considered to increase the FD-40 uptake into HepG2 cells by endocytosis. At least one of these interactive receptors binding to Ad35 shaft is degraded by heparinase 1 (Fig. 4A). Probably, it is HSPG on HepG2 cells. A second interactive receptor on K562 cells is degraded by trypsin-proteolytic enzyme, but not by heparinase 1 (Fig. 4A). Källin et al. reported that the interaction of type 35 Ad with the host-cell receptor CD46 and integrins, but not with heparin sulfate, facilitates the internalization of the vector via receptor-mediated macropinocytosis in K562 cells.13) We speculate that in certain cell types, such as the K562 cell, the shaft region of type 35 Ad seems to be involved in the transduction of Ad35 vector. This speculation might be supported by the finding that recombinant Ad35 shaft protein inhibits transduction of type 35 Ad vector in K562 cells but not in HepG2 cells (data not shown). If the functionality of the Ad shaft region in type 35 Ad infection is elucidated, we believe that it will provide useful information for virological studies.

We proved that the Ad35 shaft protein has the function of transporting macromolecules into cells by receptor-mediated endocytosis such as macropinocytosis. Macropinocytosis is part of the endocytic pathway called pinocytosis. Macropinocytosis and pinocytosis are the best studied types of receptor- and coat-independent endocytosis. Progressive macropinocytosis is mediated by a diverse group of receptors, including noncomplement-receptor integrins, lectins such as mannose receptor, the lipopolysaccharide receptor and the Caenorhabditis elegans scavenger receptor CED-1, and it is involved in the uptake of particles, bacteria and apoptotic cells.22) In macropinocytosis, these particles are released into the cytosol and an acidic pH is required for disruption of the macropinosome and pinosomal release to occur. In a transduction experiment of complex plasmids and transfection reagents into HepG2 cells, the recombinant Ad35 shaft protein significantly increased transgene expression compared to the recombinant Ad5 shaft protein (Fig. 3C). This result is similar to the experiments on the uptake of FD-40 into HepG2 cells by recombinant Ad35 shaft protein (Fig. 3A). In an immunostaining assay, after 30 min of co-treatment with FD-40 and Ad35 shaft protein, Ad35 shaft protein gradually entered into the HepG2 cells (Fig. 3B). After 8 h, the FD-40 fluorescence is weakened and is completely separately present with Ad35 shaft protein in the HepG2 cells (Fig. 3B). The decrease of FITC fluorescence intensity is thought to be the result of FITC being pH sensitive and occurring in the lysosome. Ad35 shaft proteins showing different distribution from FD-40 may escape from the endosome and penetrate into the cytoplasm. These results suggest that Ad35 shaft protein can penetrate the biological membrane and be utilized for the transport of functional particles into the cell (Fig. 3). When compared with one copy, the gene expression efficiency of type 5 Ad vector is known to be ten thousand times higher than gene transfer by a transfection reagent; part of the difference is due to intracellular trafficking.10,11) One hour after transfection of Ad vector, Ad genome revealed to be distributed to nuclear than plasmid DNA with transfection reagent, but its efficiency is about several times. The gene expression efficiency of post-nuclear translocation using an Ad vector is more than 1000 times higher than the gene expression of transfection reagent.10) The efficiency of Ad intracellular trafficking for nuclear, such as plasma membrane penetration and endosomal escape, is a function of the Ad capsid protein. Recombinant Ad35 shaft protein increases transgene expression in cells about ten-fold compared to non-protein (Fig. 3B). It was speculated from the rate of increase in transgene expression that Ad35 shaft protein improves the intracellular trafficking of plasmids to the nucleus. Conversely, Ad5 shaft or Ad5 shaft mut protein increased the uptake of FD-40 but no gain in gene expression was obtained. We checked the interaction between Ad shaft proteins and plasmid transfection complex. As a result, Ad5 shaft, Ad5 shaft mut, and Ad35 shaft protein did interact with plasmid transfection complex, but there was no quantifiable difference (data not shown). Although for that reason we do not yet have a clear answer, it is known that the physical characterization of the molecules and the compatibility of CPP are very important for intracellular introduction of macromolecules by CPP. A future investigation of the difference between the characterizations of Ad35 shaft and Ad5 shaft protein would be desirable.

Next, to identify the binding domain of the Ad35 shaft protein, we experimentally inhibited the cell binding of Ad35 shaft protein with three peptide fragments. Recombinant Ad35
shaft protein has a length of 89 amino acid residues, without bias in certain amino acid residues (Fig. 4B). We synthesized three peptide fragments, Peptides A, B and C. These are from the N-terminal 45 amino acids, C-terminal 44 amino acids and C-terminal 26 amino acids of the Ad type 35 shaft region, respectively (Fig. 4B). As a result, 30 μg/mL of peptides B and C inhibit the binding to cells of the Ad35 shaft protein in a concentration-dependent manner, because peptides B and C are one half or one quarter of the molecular weight of Ad35 shaft protein (Fig. 4C). In earlier reports by us and other researchers, the transduction efficiency of AdΔS35-L2 was reduced about ten-fold compared to conventional Ad5 vector.5,19 This is because the decrease in flexibility of the shorter shaft region reduces the secondary binding efficiency between the RGD motif of penton bases and α5-integrins. Peptide C is the C-terminal region of the Ad35 shaft protein; in the fiber protein is a portion that is in contact with the knob region, but there is no report about its function for type 35 Ad infection. We have hypothesized, from experiments on the binding of recombinant Ad35 shaft protein and peptide C to the cells, that a new molecule interacts with the shaft region of type 35 Ad. If novel features and/or interacting molecules of type 35 Ad shaft region are identified, this may contribute to the development of gene transfer vector.

We examined the cell-binding ability of peptide C and the properties of the interacting molecules on the cell surface. Interestingly, unlike the recombinant Ad35 shaft protein, peptide C did not decrease the binding ability of HSPG on HepG2 cells when pretreated with heparinase I (Figs. 4A, 5A). In K562 cells, peptide C and the recombinant Ad35 shaft protein are bound by the same trypsin-sensitive molecule on the cell surface (Figs. 4A, 5A). Greber and colleagues reported that internalization of type 35 Ad vector in K562 does not involve binding to heparin sulfate on the cell surface.13) We speculated that peptide C induces receptor-dependent endocytosis in K562 cells, and analyzed the mechanism of intracellular penetration of peptide C into K562 cells. FITC-labeled peptide C entered into K562 cells at 37°C, but not at 4°C (Fig. 5B). Considering that the induced endocytosis is temperature sensitive, a peptide C uptake inhibition experiment was conducted with Lat B and MβCD. As a result, Lat B and MβCD inhibited the uptake of peptide C into K562 cells at 37°C in a concentration-dependent manner (Fig. 5C). MβCD disrupts the raft area, which attracts functional proteins of the cell membrane, by removing the cholesterol from the cell membrane. Disruption of the raft area in cell membranes decreases an important signaling pathway of receptors by dispersing hetero-protein complexes in the raft area, not by changing the amount of proteins. The amount of peptide C bound to K562 cells did not change after treatment with MβCD compared to that without Mβ/CD, as measured by flow cytometry (data not shown). Furthermore, Lat B inhibits actin polymerization in endocytosis. Intracellular uptake of peptide C was not inhibited by chlorpromazine or 5-(N-ethyl-N-isopropyl)amiloride (EIPA), which are known as inhibitors of clathrin-dependent endocytosis and macropinocytosis, respectively (data not shown). These results suggest that the ability of peptide C to penetrate into a cell is induced by interaction between peptide C and a molecule in the membrane raft region as a result of receptor-mediated endocytosis such as pinocytosis. This molecule is a trypsin-sensitive molecule in the raft region on the membrane surface of K562 cells.

Conversely, the role of the shaft region in type 35 Ad infection has not been studied in detail in this report. As the Ad35 shaft protein did not induce macropinocytosis, it is not considered to be a major induction region in type 35 Ad infection. However, we have hypothesized that the shaft region is involved in the escape of viral particles from endosomes because the shaft protein has been found to inhibit gene expression by type 35 Ad vectors (data not shown). We believe that this hypothesis will be supported by confirming the transition of Ad35 shaft protein into the cytoplasm in a co-treatment experiment of FD-40 and Ad35 shaft protein (Fig. 3B). We hope to identify the interaction molecule of the Ad35 shaft protein and elucidate the function of the shaft region by elucidating its involvement in the type 35 Ad infection mechanism.

CPPs are collectively referred to as the cell membrane’s permeability of a peptide. CPPs are generally short peptides consisting of less than 30 amino acids and contain a polypeptide motif derived from natural proteins with penetrating functions.15) Many CPPs have been found and are used in biomedical research and as DDS. One of the major sources for CPPs are viral proteins, such as the Tat peptide from HIV, the VP22 peptide from Herpes simplex virus, and so on.16,23) Many CPPs, including HIV-Tat peptide, have many positively charged amino acids such as arginine and, when penetrating into cells, are bound to the HSPG on the cell surface.24) Furthermore, CPPs composed of hydrophobic amino acids have also been reported, and various peptides are used as CPPs.25) Peptide C has no bias in certain positively charged amino acid residues and interacts with trypsin-sensitive molecules on the surface of K562 cells. In previous studies, various CPPs had different capacities for internalizing through the plasma membrane, with some capable of penetrating bio-membranes, such as the Blood–Brain Barrier, the gastric membrane and the skin.26–28) To optimize the tissue membrane permeability by CPPs, it is necessary to use various CPPs for each tissue of the target. Although identification of the target molecule for peptide C remains a challenge for further research, we hope that peptide C will become available as a candidate molecule for DDS among a variety of CPPs.

We have identified a novel Ad-derived CPP, called peptide C, with a size of 26 amino acids, from the shaft region of type 35 Ad fiber. This novel Ad-derived CPP interacts with trypsin-sensitive molecules on the cell surface, and induces endocytosis. We suggest that, given its ability to induce receptor-dependent endocytosis in lipid rafts, this CPP might permit molecule-specific uptake. This report is the first to identify a membrane permeation peptide derived from type 35 Ad.

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Conflict of Interest The authors declare no conflict of interest.

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