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Protein Transduction Assisted by Polyethylenimine-Cationized Carrier Proteins

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Previously, we have reported that cationized-proteins covalently modified with polyethylenimine (PEI) (direct PEI-cationization) efficiently enter cells and function in the cytosol [Futami *et al.* (2005) *J. Biosci. Bioeng.* 99, 95–103]. However, it may be more convenient if a protein could be delivered into cells just by mixing the protein with a PEI-cationized carrier protein having a specific affinity (indirect PEI-cationization). Thus, we prepared PEI-cationized avidin (PEI-avidin), streptavidin (PEI-streptavidin), and protein G (PEI-protein G), and examined whether they could deliver biotinylated proteins and antibodies into living cells. PEI-avidin (and/or PEI-streptavidin) carried biotinylated GFPs into various mammalian cells very efficiently. A GFP variant containing a nuclear localization signal was found to arrive even in the nucleus. The addition of a biotinylated RNase A derivative mixed with PEI-streptavidin to a culture medium of 3T3-SV-40 cells resulted in remarkable cell growth inhibition, suggesting that the biotinylated RNase A derivative entered cells and digested intracellular RNA molecules. Furthermore, the addition of a fluorescein-labeled anti-S100C (beta-actin binding protein) antibody mixed with PEI-protein G to human fibroblasts resulted in the appearance of a fluorescence image of actin-like filamentous structures in the cells. These results indicate that indirect PEI-cationization using non-covalent interaction is as effective as the direct PEI-cationization for the transduction of proteins into living cells and for expression of their functions in the cytosol. Thus, PEI-cationized proteins having a specific affinity for certain molecules such as PEI-streptavidin, PEI-avidin and PEI-protein G are concluded to be widely applicable protein transduction carrier molecules.

Key words: cationized carrier protein, protein G, polyethylenimine, protein transduction, streptavidin.

Abbreviations: CPP, cell-penetrating peptide; DMEM, Dulbecco's modified Eagle's medium; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; eGFP, enhanced green fluorescent protein; PEI, polyethylenimine; PTD, protein transduction domain; RITC, rhodamine B isothiocyanate; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Protein delivery into living cells has been attracting a great deal of attention as a novel technology having potential both for basic research in cellular biology and for therapeutic application. Short basic peptides named protein transduction domains (PTD) or cell-penetrating peptides (CPP) have been found to deliver molecular cargo to target cells within the cytoplasm and/or nucleus of living cells. These peptides corresponding to the human immunodeficiency virus type 1 Tat-(48–60) (*1*, *2*), Antennapedia-(43–58) (*3*, *4*) and poly-Arg (*5*) are among the most well known PTD or CPP. By attaching these carrier peptides, efficient protein transduction of $p27^{Kip1}$ (*6*), p53 (*7*), Bcl-xL (*8*, *9*), Cre recombinase (*10*, *11*), and HOXB4 (*12*, *13*) was demonstrated to enable the modulation of cellular events *in vitro* and/or *in vivo*. The mechanism by which these cell-penetrating peptides (and their conjugates) enter cells has not yet been determined, but the electrostatic interaction between these peptides with polycationic charges and negatively charged proteoglycans on the cell surface is believed to trigger their efficient transduction (*14*, *15*).

As for protein cationization itself, it was proposed more than a decade ago that the method is applicable to intracellular protein delivery *via* adsorption-mediated endocytosis (*16*, *17*). The cationization of proteins is accomplished by extensive amidation of carboxyl groups with

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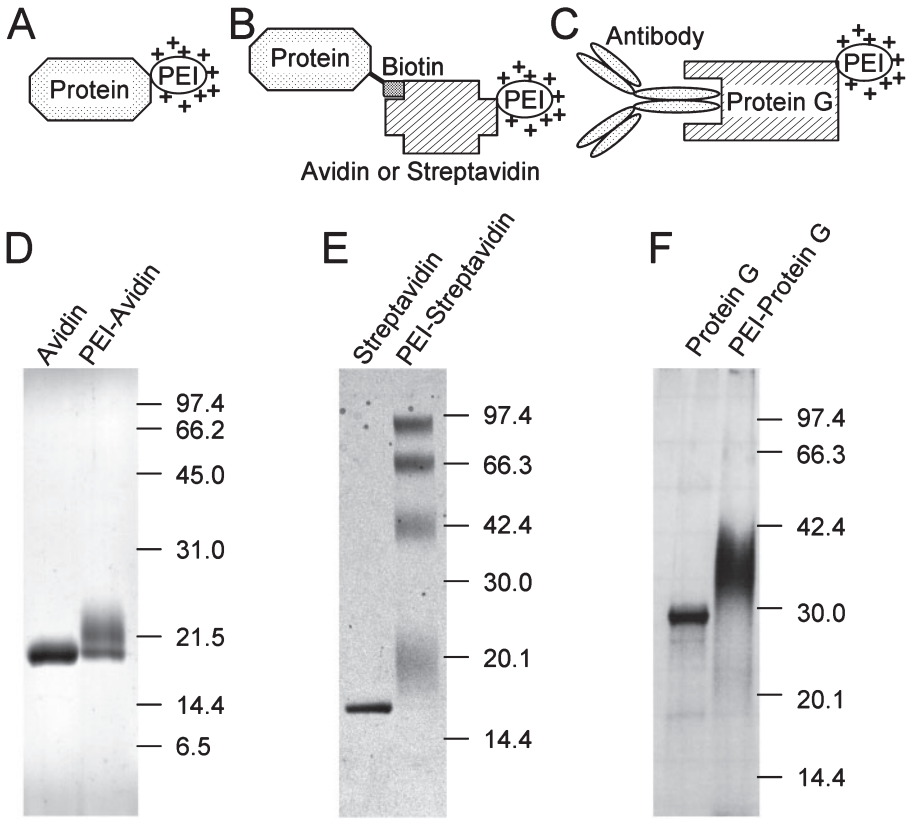

Fig. 1. **Schematic drawing of variously cationized proteins and SDS-PAGE analysis of native and chemically modified avidin, streptavidin, and protein G.** Protein cationized directly with PEI by carbodiimide reaction (A). Biotinylated protein complexed with PEI-avidin or PEI-streptavidin (B). Antibody bound to PEI-protein G (C). PEI-modification of avidin (D), streptavidin (E), and protein G (E) was analyzed by SDS-PAGE under reducing conditions in 15% (D), 15–25% gradient (E) and 10–20% gradient (F) polyacrylamide gels.

various diamines (*e.g.*, ethylenediamine). In fact, we have demonstrated that non-toxic secretory RNases become cytotoxic by cationization with ethylenediamine, indicating that they acquired a new function to pass through the cell membrane, although the cationized RNases showed considerably decreased enzymatic activity because of the modification of many carboxyl groups required for sufficient cationization (*18*). Thus, cationization is considered to be a powerful strategy for promoting cellular uptake of proteins. However, unfavorable effects on protein function and stability due to the extensive modification of carboxyl groups with diamines may limit its application (*19*).

Polyethylenimine (PEI) is a macromolecule with the highest cationic density among existing polycationic polymers. The modification of only a few residues with PEI (limited chemical conjugation) is needed for sufficient cationization to deliver proteins into living cells (Fig. 1A); therefore, the protein function can be maintained to a greater degree by limited PEI-cationization than by extensive diamine-cationization. Furthermore, PEI is toxicologically safe and has been used as a food additive, suggesting PEI to be a more suitable reagent than diamines for the cationization of proteins. In this context, we have demonstrated PEI-cationization to be an extremely useful method for protein transduction. Namely, we succeeded by using PEI-cationization to achieve efficient transduction of RNase A (expressing cytotoxicity), eGFP (intracellular fluorescence) (*20*) and anti-S100C (beta-actin binding protein) antibody (imaging of S100C along actin filaments and neutralization of S100C function) (*20*, *21*) into living cells. PEI-cationized proteins showed the ability to be transduced into living cells *in vitro* with 100% efficiency and at a substantially increased level compared with current PTD-fusion proteins (*20*). Therefore, PEI-cationization is considered to be an alternative and promising protein transduction method. However, the rather tedious chemical modification may limit its application. Thus, it may be more convenient if a protein could be delivered into cells just by mixing the protein with a PEI-cationized carrier protein having a specific affinity (indirect PEI-cationization) instead of by covalent modification with PEI (direct PEI-cationization; Fig. 1A). We describe here the utility of this 'PEI-cationized carrier protein method' for protein transduction into living cells using the following combinations as models: biotinylated GFP and biotinylated RNase A derivative with PEI-modified avidin or streptavidin (PEI-avidin or PEI-streptavidin, Fig. 1B) and anti-S100C antibody with PEI-modified protein G (PEI-protein G, Fig. 1C).

EXPERIMENTAL PROCEDURES

Materials—PEI (molecular mass 600) was purchased from Wako Chemicals (Osaka). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and Sulfo-NHS-SS-biotin was purchased from Pierce (Rockford, IL, USA). Bovine RNase A (Type XII-A), protein G and RITC-labeled rabbit anti–mouse IgG(H+L) polyclonal antibody (RITC–anti-mouse IgG) were obtained from Sigma (St. Louis, MO, USA). Biotin-AC_5-OSu was purchased from Dojindo (Kumamoto, Japan). Gradient polyacrylamide gel was purchased from Daiichi Pure Chemicals (Tokyo, Japan). An RNase A derivative (RNaseA-SO_3), in which the carboxyl groups of RNase A

were extensively amidated with taurine, was prepared as described previously (*18*). The preparation of rabbit antibody against human S100C was described previously (*22*). A complementary DNA of His-tagged eGFP, whose sequence has been reported previously (*6*), was subcloned into the pET14b vector (Novagen, WI, USA). A complementary DNA of GFPNuc, which encodes His-tagged eGFP with three copies of the nuclear localization signal (NLS) of the simian virus 40 large T-antigen fused at its C-terminus, was subcloned into the pET14b vector. The expression vector of His-tagged GFPUV5, a variant GFP with stronger fluorescence than eGFP, was kindly provided by Dr. M. Suzuki (*23*). These fusion proteins expressed in *Escherichia coli* BL21 (DE3) pLysS at 25°C were purified as described previously (*20*). Directly PEI-cationized eGFP was prepared by the carbodiimide reaction as described elsewhere (*20*).

PEI-Modification—Coupling reactions of proteins with PEI by EDC were carried out as described previously (*18*, *20*) in order to obtain PEI-cationized streptavidin (PEI-streptavidin), avidin (PEI-avidin) and protein G (PEI-protein G). The coupling conditions employed for each were as follows: streptavidin (0.1 mg/ml) in PEI-solution (60 mg/ml, pH 5) and 0.3 mg/ml EDC for 16 h at room temperature; avidin (2 mg/ml) in PEI-solution (100 mg/ml, pH 5) and 0.1 mg/ml for 1 h at room temperature; protein G (0.5 mg/ml) in PEI-solution (60 mg/ml, pH 5) and 1 mg/ml EDC for 16 h at 4°C. After the reaction, each solution except streptavidin was exhaustively dialyzed against PBS and stored at 4°C (avidin) or –20°C (protein G) until use. In the case of streptavidin, the solution was dialyzed against distilled water and then against 0.5 M hydroxylamine solution (pH 7.5). After 12-h dialysis against hydroxylamine, the solution was exhaustively dialyzed against distilled water and lyophilized. The lyophilized PEI-streptavidin was dissolved in PBS when it was used.

Biotinylation—Biotinylated eGFP (biotin-eGFP) or RNaseA-SO_3 (biotin-RNaseA-SO_3) was prepared by mixing a protein solution (pH adjusted to 9 with dilute NaOH) with Biotin-AC_5-OSu dissolved in dimethylformamide (DMF) at a molar ratio of the biotinylating reagent : protein of 2:1. GFPUV5 and GFPNuc were also biotinylated with Biotin-$(AC_5)_2$-OSu or Sulfo-NHS-SS-biotin in DMF at a molar ratio of the biotinylating reagent : protein of 5:1 to give biotin-GFPUV5 or biotin-SS-GFPUV5, and biotin-GFPNuc or biotin-SS-GFPNuc, respectively. After 2-h incubation at room temperature, the biotinylated proteins were purified by PD10 gel filtration (Amersham Bioscience, NJ, USA) using PBS as an elution buffer.

Cell Culture and Fluorescence Microscopy—BALB/c 3T3, 3T3-SV-40 (Dainippon Pharmaceutical Co., Tokyo), human fibroblasts OUMS-36 (*24*), HEK293 PEAK^{rapid} (Edge Biosystems, Gaithersburg, ML, USA), NIH-3T3, and HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 70 μg/ml of kanamycin. K562 cells were grown in RPMI1640 medium supplemented with 10% FBS and 70 μg/ml of kanamycin. Human fibroblasts (HFL-1) were cultured in Ham's F-12 medium supplemented with 15% FBS and 0.1 mg/ml streptomycin. Confocal microscopic analysis was performed by the following procedure. First, OUMS-36 or HFL-1 cells were grown in a glass-base dish in growth medium at 37°C until 50% confluent. Then the medium was changed to serum-free medium and protein samples were added to the medium. After 3-h incubation, the cells were washed several times with PBS and observed directly under a confocal laser-scanning microscope (model MRC-1024; Bio-Rad, CA, USA or model LSM 510; Carl Zeiss, Jena, Germany). In other cases, cells were grown on cover glasses in each growth medium at 37°C until 70% confluent. Then protein samples were added to the medium in the presence or absence of 10% FBS. After incubation for the indicated periods, the cells were washed several times with PBS and observed directly under a confocal laser-scanning microscope (model LSM 510; Carl Zeiss, Jena, Germany).

Flow Cytometry—Various exponentially growing cells were incubated with 100 nM of each of biotin-GFPUV5 and PEI-avidin that were premixed in the growth medium. After incubation for 24 h at 37°C, the cells were washed with PBS, dissociated with trypsin, and analyzed by fluorescence flow cytometry (FACSCalibur; Becton Dickinson, NJ, USA).

Cytotoxicity Assays—Cytotoxicity assays were conducted with 3T3-SV-40 cells. Cell viability was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) as described previously (*25*, *26*) with some modification. Briefly, exponentially growing 3T3-SV-40 cells were seeded in a 96-well plate (4,000 cells/well) and incubated for 24 h at 37°C. The medium was replaced with serially diluted protein samples at the indicated concentrations (10 nM–1 μM) in serum-free medium. After 4-h cultivation, the medium was replaced with DMEM containing 10% FBS. Incubation was continued for 2 days before termination, and cell viability was determined using the MTT assay according to the manufacturer's instructions. The percent of cell growth was calculated in triplicate using cells grown without protein samples as a control.

RESULTS

Preparation of PEI-Carrier Proteins—Streptavidin, avidin and protein G were used as model carrier proteins for protein transduction. These proteins were cationized with PEI (average molecular mass 600) by a carbodiimide reaction under the conditions described in "EXPERIMENTAL PROCEDURES." To confirm PEI-modification, native and PEI-modified proteins were analyzed by SDS-PAGE. PEI-modified proteins gave bands that migrated more slowly and were broader in shape than the unmodified proteins (Fig. 1, D, E, and F). Avidin and streptavidin are both tetrameric proteins. In the case of PEI-avidin, about 2/3 of the monomer units (corresponding to 8/3 of a tetramer unit) seemed to be modified with PEI as judged from the ratio of unmodified monomer (Fig. 1D). On the other hand, PEI-streptavidin did not contain unmodified monomers, but contained non-dissociable dimers, trimers and tetramers in addition to modified monomers (Fig. 1E). These non-dissociable oligomers might be covalently cross-linked among intersubunits by the carbodiimide reaction. These results indicate that avidin, streptavidin and protein G were all successfully cationized with PEI, although avidin was somewhat lightly cationized. These

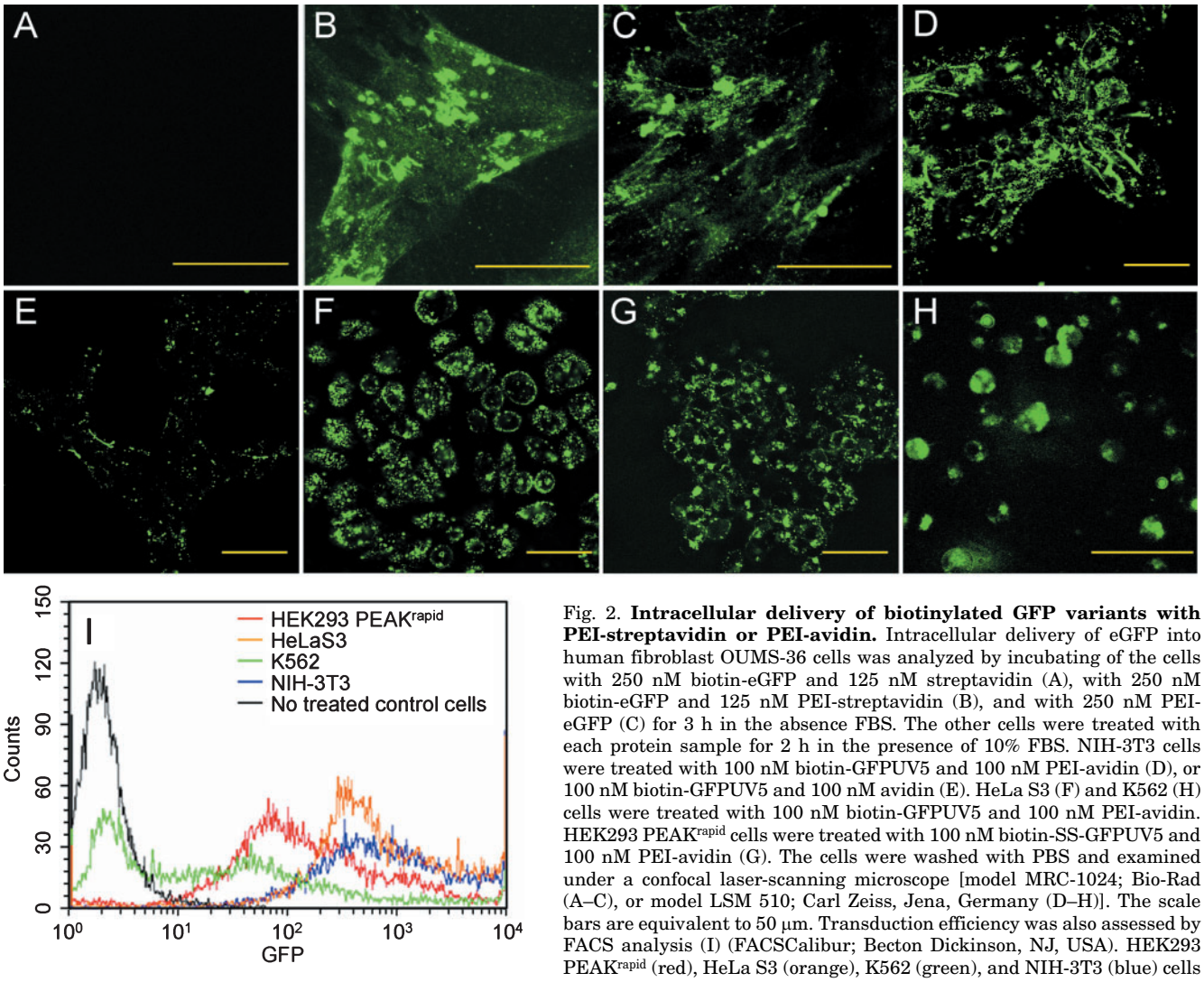

Fig. 2. **Intracellular delivery of biotinylated GFP variants with PEI-streptavidin or PEI-avidin.** Intracellular delivery of eGFP into human fibroblast OUMS-36 cells was analyzed by incubating of the cells with 250 nM biotin-eGFP and 125 nM streptavidin (A), with 250 nM biotin-eGFP and 125 nM PEI-streptavidin (B), and with 250 nM PEI-eGFP (C) for 3 h in the absence FBS. The other cells were treated with each protein sample for 2 h in the presence of 10% FBS. NIH-3T3 cells were treated with 100 nM biotin-GFPUV5 and 100 nM PEI-avidin (D), or 100 nM biotin-GFPUV5 and 100 nM avidin (E). HeLa S3 (F) and K562 (H) cells were treated with 100 nM biotin-GFPUV5 and 100 nM PEI-avidin. HEK293 PEAK^{rapid} cells were treated with 100 nM biotin-SS-GFPUV5 and 100 nM PEI-avidin (G). The cells were washed with PBS and examined under a confocal laser-scanning microscope [model MRC-1024; Bio-Rad (A–C), or model LSM 510; Carl Zeiss, Jena, Germany (D–H)]. The scale bars are equivalent to 50 μm. Transduction efficiency was also assessed by FACS analysis (I) (FACSCalibur; Becton Dickinson, NJ, USA). HEK293 PEAK^{rapid} (red), HeLa S3 (orange), K562 (green), and NIH-3T3 (blue) cells were treated with 100 nM of biotin-GFPUV5/PEI-avidin for 24 h in the presence of 10% FBS. One of the non-treated cells (HEK293 PEAK^{rapid}) is indicated by the black line as a representative.

PEI-modified proteins did not show any remarkable cytotoxicity toward tested mammalian cells at concentrations below 10 μM (data not shown), and were used as PEI-carrier proteins in this study.

Transduction of Biotinylated GFP with PEI-Streptavidin or with PEI-Avidin—The first model study of protein transduction assisted by PEI-cationized carrier protein was that using streptavidin-biotin or avidin–biotin interaction. eGFP variants were selected as model proteins to be delivered into cells because their cellular uptake can be monitored by fluorescence. Biotin-eGFP was mixed with PEI-streptavidin at a molar ratio of 1 to 2, and the mixture was added to a culture medium, serum-free DMEM, of OUMS-36 fibroblasts at a biotin-eGFP concentration of 250 nM. After incubation for 3 h at 37°C, the cells were washed with PBS and observed under a confocal laser-scanning microscope. The fluorescence of eGFP was clearly detected in these cells (Fig. 2B). There were no remarkable differences in the distributions and intensities of fluorescence of eGFP between cells treated with the biotin-eGFP/PEI-streptavidin complex (Fig. 2B) and those treated with directly PEI-cationized eGFP (PEI-eGFP, Fig. 2C). On the other hand, eGFP fluorescence could not be detected in cells treated with the biotin-eGFP/streptavidin (PEI-null) complex (Fig. 2A). These results indicate that in order to internalize proteins into living cells, indirect PEI-cationization using biotin-streptavidin interaction is as effective as direct PEI-cationization.

It has been previously reported that native avidin, which is a cationic protein having a net charge of +24 per tetramer at neutral pH, has the ability to introduce biotinylated peptides and antisense oligonucleotides into cells (*27*). However, PEI-cationized avidin (estimated net charge to be about +70 per tetramer) carries biotin-GFPUV5 into NIH-3T3 fibroblast cells much more efficiently than native avidin (Fig. 2, D and E), indicating that additional cationization of avidin does improve the transduction efficiency of biotinylated proteins. The transduction of biotinylated GFPUV5 (biotin-GFPUV5 or

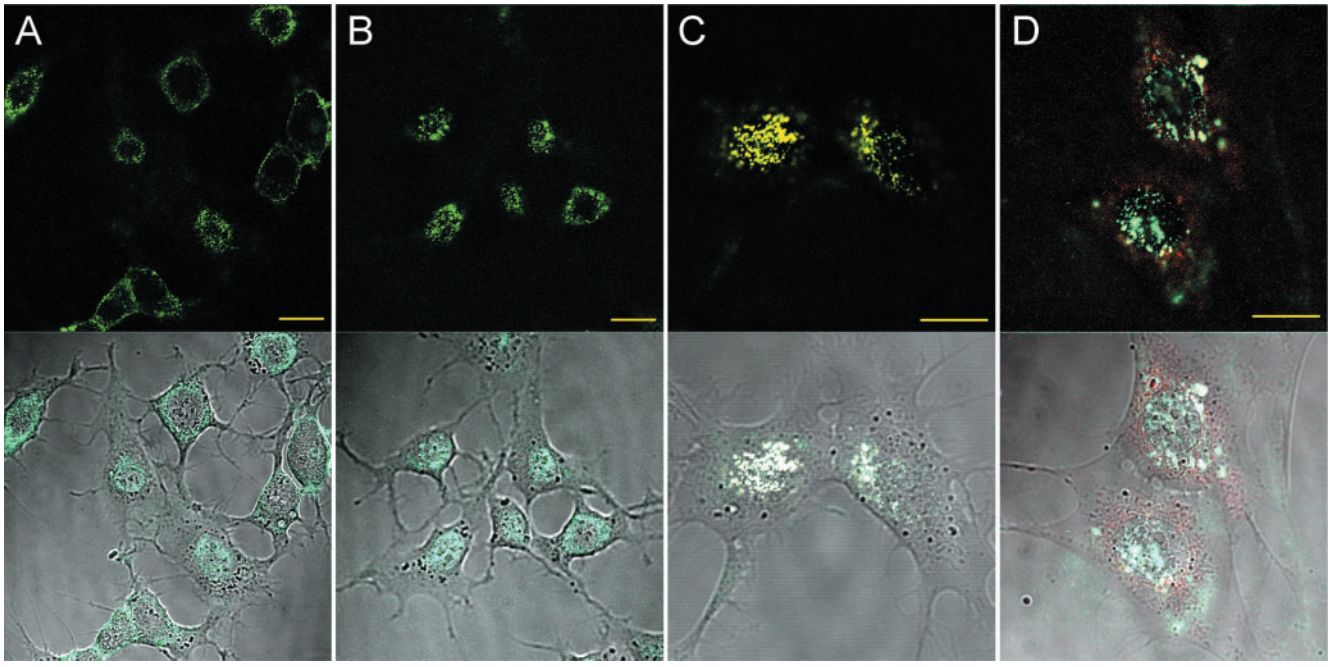

Fig. 3. **Confocal laser-scanning microscopy of cellular biotinylated GFPNuc fluorescence.** Intracellular delivery of GFP variants into Balb/c 3T3 cells was analyzed by incubatiing of the cells with 50 nM biotin-GFPUV5 and 50 nM PEI-avidin (A) and with 50 nM biotin-GFPNuc and 50 nM PEI-avidin (B) for 1 h in the absence FBS. Cells were treated with 100 nM biotin-GFPNuc and 100 nM PEI-avidin-RITC (C), or 100 nM biotin-SS-GFPNuc and 100 nM PEI-avidin-RITC (D) for 3 h in the absence of 10% FBS. The cells were washed with PBS, and examined under a confocal laser-scanning microscope (model LSM 510; Carl Zeiss, Jena, Germany). The scale bars are equivalent to 50 μm.

biotin-SS-GFPUV5) with PEI-avidin succeeded not only in fibroblast cell lines, but also in an epithelial cell line HeLa S3 (human cervical cancer cell line) (Fig. 2F), human embryonic kidney cell line HEK293 PEAK^{rapid} cells (Fig. 2G), and K562 (human chronic myelogenous leukemic cells) (Fig. 2H). Although biotin-SS-GFPUV5 contains a cleavable disulfide bond between the biotin moiety and the protein portion, Fig. 2G indicates that the cleavage of the disulfide bond did not occur outside the cells in the medium. FACS analysis revealed that biotinylated GFPUV5 was introduced with PEI-avidin into all of NIH-3T3 and HeLa S3 cells but only into about 65% of K562 cells (Fig. 2I).

Biotinylated GFP Fused with a Nuclear Localization Signal Sequence Was Carried into Cells by PEI-Avidin and Translocated into the Nucleus—Biotin-GFPUV5 transduced into NIH-3T3 cells by PEI-avidin produced granular fluorescence images around the inner rim of each cell (Fig. 3A). Fluorescence was hardly observed in nuclei. The granular images may indicate the partial localization of biotin-GFPUV5 in endocytic vesicles, as suggested previously that PEI-cationized proteins are internalized into cells mainly through an endocytic internalization pathway (*20*). On the other hand, biotin-GFPNuc, a biotinylated GFP derivative fused with three copies of the nuclear localization signal sequence (NLS) of the SV40 large T-antigen at the C-terminus, resulted in fluorescence in most nuclei (Fig. 3B). When biotin-GFPNuc was introduced into cells with rhodamine labeled PEI-avidin (PEI-avidin-RITC), green fluorescence due to GFP and red fluorescence due to RITC were co-localized in nuclei (Fig. 3C), suggesting that biotin-GFPNuc entered the nucleus as a complex with PEI-avidin-RITC. These results indicate that biotinylated proteins having NLS complexed with PEI-avidin entered not only endosomal vesicles but also the nucleus through the cytosol. Biotin-SS-GFPNuc, which has a cleavable disulfide bond between biotin and GFPNuc, was also introduced with PEI-avidin-RITC into cells. As shown in Figure 3D, green fluorescence from GFPNuc tended to appear in the nucleus while red fluorescence from PEI-avidin-RITC remained in the cyotosol. These results indicate that when a complex of biotin-SS-GFPNuc with PEI-avidin-RITC enters the cytosol, the disulfide bond is cleaved under the reducing environment causing the dissociation of GFPNuc from PEI-avidin-RITC, and GFPNuc portion is then further translocated into the nucleus.

RNase A Derivatives Carried to the Cytosol by PEI-Streptavidin Show Cytotoxicity—RNase A was selected as another model protein to be delivered into cells by indirect PEI-cationization using streptavidin-biotin interaction. We previously reported that RNaseA-SO_3, an RNase A derivative in which the carboxyl groups are extensively modified with taurine in amide bonds, acquires resistance to an RNase inhibitor and exhibits cytotoxicity by long-term exposure to cells due to endocytic leakage into the cells followed by cellular RNA degradation (*18*). Therefore, biotinylated RNaseA-SO_3 (biotin-RNaseA-SO_3) was prepared to examine whether PEI-streptavidin could intensify its cytotoxicity. 3T3-SV40 cells were cultured overnight at 37°C, and the medium was changed to serum-free DMEM. Biotin-RNaseA-SO_3 and PEI-streptavidin were mixed at a same molar ratio, and the mixture was added to the culture medium at biotin-RNaseA-SO_3–

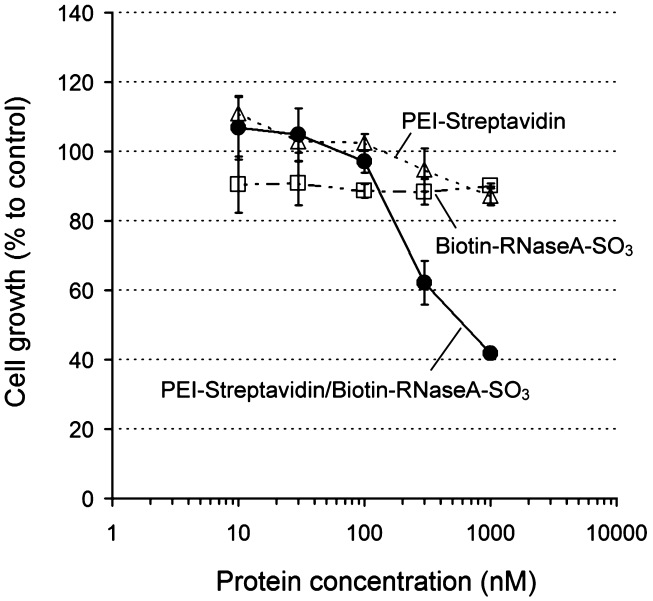

Fig. 4. **Cytotoxic activity of RNaseA-SO_3 introduced into cells using PEI-streptavidin carrier against 3T3-SV-40 cells.** 3T3-SV-40 cells in DMEM supplemented with 10% FBS were seeded into a 96-well plate (4000 cells/well), left to adhere for 24 h, and then treated with various concentrations of each protein sample in serum-free medium for 4 h. The culture medium has been replaced with DMEM containing 10% FBS, and the cells were cultured for 3 days before cell growth in each well was monitored by the MTT assay. Each value is the mean of three cultures, and is presented as a percentage of the value for buffer-treated cells, which is the mean value of the medium without protein sample.

based concentrations of 10 to 1,000 nM. After cultivation for 4 h, the medium was replaced with DMEM containing 10% FBS. The cells were cultured for another 2 days and then the viability of the cells was measured by MTT assay. As shown in Fig. 4, a dose-dependent cytotoxic effect of the biotin-RNaseA-SO_3/PEI-streptavidin complex was clearly observed (IC_{50} = 0.4 μM), while neither biotin-RNaseA-SO_3 nor PEI-streptavidin showed cytotoxic effects by themselves under the conditions employed. As mentioned before, PEI-carrier proteins, including PEI-streptavidin, do not show any remarkable cytotoxicity to the examined mammalian cells at concentrations below 10 μM. These results indicate that biotin-RNaseA-SO_3 is efficiently introduced into cells by the assistance of PEI-streptavidin and allowed to express RNase activity in cells resulting in the expression of cytotoxic activity.

Transduction of IgG Using PEI-Protein G—The second model study of protein transduction by indirect PEI-cationization was that using immunoglobulin-protein G interaction. An S100C protein belonging to the EF-hand Ca^{2+}-binding protein family was a key mediator of growth arrest (*21*). In a non-confluent state, S100C protein kept binding to actin filaments in normal human fibroblasts. Previously, we have reported that the FITC-labeled and directly PEI-cationized anti-S100C antibody enters cells producing fluorescence images of actin-like filamentous structures in human normal fibroblasts in a non-confluent state (*20*). Therefore, the anti-S100C antibody was selected as a model protein to be delivered into cells by PEI-protein G. The FITC-labeled anti-S100C antibody was mixed with PEI-protein G (1:1 molar ratio), and the mixture was added to the serum-free F-12 culture medium of human normal fibroblast HFL-1 cells at 100 nM. After 3-h cultivation, the cells were washed with PBS and observed under a confocal laser-scanning microscope. In addition to the fluorescence images of endosome-like granular structures, those of actin-like filamentous structures were observed in the cytoplasmic region (Fig. 5A). On the other hand, only granular structures were observed in cells treated with RITC-anti-mouse IgG/PEI-protein G complex (Fig. 5B). These results indicate that the PEI-protein G delivered the anti-S100C antibody into cells, which then at the right positions, that is, on S100C proteins bound to actin filaments.

DISCUSSION

We examined here the transduction efficiency of several proteins carried by PEI-cationized carrier proteins, PEI-streptavidin, PEI-avidin and PEI-protein G. When human OUMS-36 fibroblasts were incubated with the biotin-eGFP/PEI-streptavidin complex in serum-free DMEM, the cells exhibited granular fluorescence images in the cytoplasm rather than in the nuclei (Fig. 2B). Similar fluorescence images were observed in cells treated with eGFP cationized directly with PEI (Fig. 2C). Human HFL-1 normal fibroblasts treated with an RITC–anti-mouse IgG/PEI-protein G complex also gave similar granular fluorescence images in the cytoplasm (Fig. 4B). Furthermore, cells treated with RITC–anti-mouse IgG/PEI-cationized protein A, and those treated with a complex of RITC–anti-mouse IgG and PEI-cationized goat anti-mouse IgG also gave similar images (data not shown). On the other hand, no fluorescence was observed in cells treated with the biotin-eGFP/streptavidin (PEI-null) complex (Fig. 2A). PEI-avidin also effectively introduced biotin-GFPUV5 into cells (Fig. 2D) compared with avidin (PEI-null) (Fig. 2E). The transduction of biotinylated GFPUV5 with PEI-avidin was successful in all examined adhesive cell lines (Fig. 2, D, F, and G), although the efficiency differed depending on the cell line (Fig. 2I). Even in the case of a floating cell line, K562, biotin-GFPUV5 was introduced in about 65% of total cells by PEI-avidin (Fig. 2, H and I). Essentially, these PEI-carriers did not show any cytotoxicity at concentrations below 10 μM (data not shown). These results clearly indicate that the indirect cationization of proteins with PEI utilizing non-covalent intermolecular interactions, such as biotin-streptavidin, biotin-avidin, and antibody–protein G, as well as antibody-protein A and antigen-antibody, is as effective for protein transduction into living cells as direct cationization with PEI utilizing covalent conjugation.

In order to examine whether proteins thus internalized into cells could reach the cytosol and exhibit their functions inside cells, biotin-GFPNuc, biotin-RNaseA-SO_3 and FITC-labeled anti-S100C antibody were internalized into living cells by PEI-avidin, PEI-streptavidin and PEI-protein G, respectively. As a result, biotin-GFP-Nuc was imported into the nucleus (Fig. 3B), biotin-RNaseA-SO_3 expressed increased cytotoxicity due to the effective degradation of intracellular RNA molecules (Fig. 4), and FITC-labeled anti-S100C antibody revealed

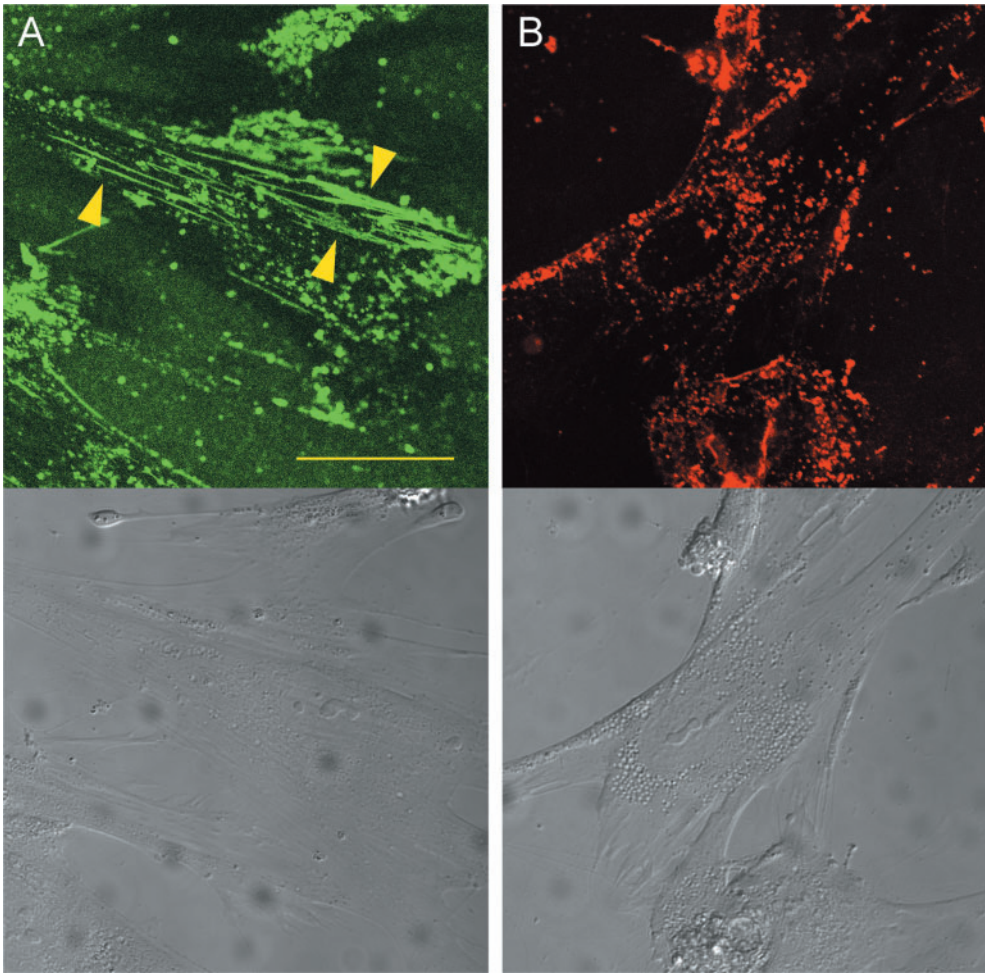

Fig. 5. **Confocal laser-scanning microscopy of the distribution of FITC-labeled anti-S100C antibodies introduced into cells by PEI-cationized protein G.** HFL-1 cells were grown in glass-base dishes in the presence of 15% FBS at 37°C until fifty percent confluent. Then the medium was replaced with serum-free medium and protein was added to the medium. After 3-h incubation, the cells were washed with PBS several times and analyzed directly under a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Jena, Germany). When cells were treated with FITC–anti-S100C IgG/PEI-protein G complex, fluorescence was detected along with actin filaments (A), whereas no such specific fluorescence pattern was detected in cells treated with RITC–anti-mouse IgG/PEI-Protein G complex (B). Lower panels are differential interference contrast images corresponding to the upper panels. Arrowheads indicate filamentous structures. The scale bar is equivalent to 50 μm.

fluorescence images of filamentous structures due to binding to S100C proteins bound to actin filaments (Fig. 5A). All of these results clearly indicate that proteins internalized into living cells by indirect PEI-cationization can exhibit their functions inside cells as well as those internalized into cells by direct PEI-cationization.

Previously, we have demonstrated that PEI-cationization of proteins by direct modification with PEI is a very efficient method of protein transduction, and is, therefore, a very powerful tool for investigating cellular functions of proteins (*20*). In the present study, we succeeded in expanding of PEI-cationization to a method in which proteins to be delivered into living cells are PEI-cationized by "PEI-labeled carrier proteins." Once PEI-carrier proteins have been prepared, direct chemical modification with PEI of proteins to be delivered into cells is not necessary; only mixing the intact protein with its PEI-antibody, antibody with PEI-protein G or A, and biotinylated protein with PEI-streptavidin or PEI-avidin, is needed. Therefore, the tedious PEI modification step can be avoided. This methodology will also be effective if there are many proteins to be examined, such as in the case of screening the cellular functions of numerous proteins. For example, using PEI-streptavidin or PEI-avidin as a carrier protein, any biotinylated protein can be internalized into cells. Biotinylation is one of the most-extensively studied chemical modifications and many kinds of biotinylating reagents are currently available. Therefore, biotinylation may be easier and safer with regard to the risks of conformational change and loss of function of a protein than direct PEI-modification. Besides chemical modification, sequence-specific biotinylation with biotin holozyme synthetase is also available by co-expression of proteins with the biotinylation sequence and enzyme using an *E. coli.* expression system (*28*). PEI-protein G and PEI-protein A make it possible to internalize valuable and rare antibodies into living cells. This method may be used for the neutralization of intracellular protein function if a neutralizing antibody is available.

Disulfide bonds can be cleavaged under the reductive environment of the cytosol. Therefore, by using biotinylating reagents having disulfide bonds, a protein delivered into cells would become free from the PEI-carrier protein in the cytosol. In fact, the transduction of biotin-SS-GFPNuc with PEI-avidin resulted in the dissociation of GFPNuc from PEI-avidin within cells (Fig. 3D). This type of biotinylating reagent may be effective for the transduction of a protein if cationic modification interferes with the protein function.

A previous report suggested that native avidin has the ability to introduce peptides and antisense oligonucleotides into cells (*27*). However, as shown in Fig. 2, D and E, PEI-avidin is highly superior to intact avidin. It has also been reported that a fluorescence-labeled antibody

can be introduced into cells using TAT-protein A as a carrier in which protein A is genetically fused with the TAT peptide (*29*). However, the expression of the functions of the proteins transduced into cells by these carrier proteins was not clear in either report. Therefore, this is the first report of proteins introduced by carrier proteins reaching the cytosol and expressing their function inside cells: RNase activity, transport to the nucleus, and recognition of antigen S100C.

In addition to filamentous images, granular fluorescence images were observed in cells treated with either PEI–anti-S100C antibody (*20*) or anti-S100C antibody/PEI–protein G complex (Fig. 5A). PTD- or CPP-fused proteins mediate cell surface adhesion, which is followed by internalization into cells by endocytosis, and fluorescence-labeled TAT-fusion proteins localize in endocytic vesicles (*11*, *30*). We have also reported that PEI-cationized eGFP is internalized through an endocytic internalization pathway (*20*). The granular fluorescence images suggest the partial localization of excess PEI–anti-S100C or anti-S100C antibody/PEI-protein G complex in endocytic vesicles. Otherwise, the anti-S100C antibody used here contained non-specific IgGs. For live cell imaging of intracellular proteins, these granular fluorescence images are not preferable. Therefore, part of the future research will seek to reduce these granular fluorescence images.

In conclusion, the "PEI-cationized carrier method" developed here is a simple and widely applicable protein transduction method, and is expected to serve as a potential tool for basic research in cellular biology and for therapeutic applications

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