J. Biochem. **144,** 447–455 (2008)
doi:10.1093/jb/mvn087

Intracellular Delivery of Glutathione *S*-transferase-fused Proteins into Mammalian Cells by Polyethylenimine–Glutathione Conjugates

Hitoshi Murata[1], Junichiro Futami[1,2], Midori Kitazoe[1], Takayuki Yonehara[1], Hidetaka Nakanishi[3], Megumi Kosaka[1], Hiroko Tada[1], Masakiyo Sakaguchi[4], Yasuyuki Yagi[3], Masaharu Seno[1,5], Nam-ho Huh[4] and Hidenori Yamada[1,2,*]

[1]Department of Bioscience and Biotechnology, Faculty of Engineering, Graduate School of Natural Science and Technology; [2]Engineering Innovation Center, Okayama University, Okayama 700-8530; [3]Nippon Shokubai Co., Ltd., 5-8 Nishi Otabi-cho, Suita, Osaka 564-8512; [4]Department of Cell Biology, Graduate School of Medicine and Dentistry, Okayama University, Okayama 700-8558; and [5]Research Center for Biomedical Engineering, Okayama University, Okayama 700-8530, Japan

Received March 28, 2008; accepted June 27, 2008; published online July 4, 2008

The glutathione *S*-transferase (GST)-fused protein expression system has been extensively used to generate a large quantity of proteins and has served for functional analysis *in vitro*. In this study, we developed a novel approach for the efficient intracellular delivery of GST-fused proteins into living cells to expand their usefulness up to *in vivo* use. Since protein cationization techniques are powerful strategies for efficient intracellular uptake by adsorptive-mediated endocytosis, GST-fused proteins were cationized by forming a complex with a polycationic polyethylenimine (PEI)–glutathione conjugate. On screening of protein transduction, optimized PEI–glutathione conjugate for protein transduction was characterized by a partly oligomerized mixture of PEI with average molecular masses of 600 (PEI600) modified with multiple glutathiones, which could have sufficient avidity for GST. Furthermore, enhanced endosomal escape of transduced GST-fused proteins was observed when they were delivered with a glutathione-conjugated PEI600 derivative possessing a hydroxybutenyl moiety. These results were confirmed by both intracellular confocal imaging of GST-fused green fluorescent protein and activation of an endogenous growth signal transduction pathway by a GST-fused constitutively active mutant of a kinase protein. These PEI-glutathione conjugates seem to be convenient molecular tools for protein transduction of widely used GST-fused proteins.

Key words: cationization; glutathione; glutathione *S*-transferase; polyethylenimine; protein transduction.

Abbreviations: caMEK1, constitutively active mitogen-activated protein kinase kinase 1; EGF, epidermal growth factor; ERK1, mitogen-activated protein kinase 1; FBS, fetal bovine serum; GST, glutathione *S*-transferase; MBP, myelin basic protein; PEI, polyethylenimine; PI, propidium iodide; THF, tetrahydrofuran.

Over the past decade, techniques for protein transduction into living cells have been developed as tools for controlling cellular function, and they are recognized as a novel methodology in pharmaceutical research (*1*–*7*). Currently, the most commonly used protein transduction method utilizes cationic peptides known as cell-penetrating peptides (CPPs). Although the internalization mechanisms of CPP-fused proteins remain controversial, it is now becoming clear that the main port of entry into cells is electrostatic adsorptive-mediated endocytosis (*8*, *9*). This is because the living cell surface is being negatively charged. Major sources of the negative charge on the cellular surface are glycosaminoglycans (GAGs) attaching to the extracellular domain in membrane proteins. In this point of view, chemical protein cationization techniques offer a powerful alternative protein transduction strategy by adsorptive-mediated endocytosis (*1*, *10*–*17*). Recent studies have revealed that a cationic polymer of polyethylenimine (PEI) is a suitable material for making proteins highly cationic with minimum effects on protein functions and with minimal cytotoxicities (*1*, *14*, *15*, *17*).

In order to perform protein transduction experiments, expression and purification of a recombinant protein are usually required. An *Escherichia coli* expression system is convenient and inexpensive, and it has therefore been extensively used for recombinant protein production in most laboratories. To facilitate purification, proteins of interest are often fused to affinity tags. Glutathione *S*-transferase (GST) is one of the most extensively used affinity tags. A GST-fusion system allows functional domains or full length of proteins to be designed as proteins fused to the C-terminus of *Schistosoma japonicum* GST (*18*). Generally, the GST-fused protein expression system shows relatively higher possibility to give soluble proteins than other fusion systems (*19*) and the proteins

*To whom correspondence should be addressed. Fax: +81-86-251-8215, E-mail: yamadah@cc.okayama-u.ac.jp

expressed are easily purified by glutathione-immobilized affinity chromatography from soluble fractions of cell lysate. Although proteins without a GST-tag can be prepared by treatment with a protease that specifically recognizes and cleaves the linker region (*18*), many GST-fused proteins are sufficient by themselves for functional analysis. According to a high reliability of GST-fusion system, there are large numbers of research resource of GST-fused expression plasmid DNA.

In this study, we developed a novel convenient tool for efficient GST-fused protein transduction into living cells by the formation of a complex of a GST-fused protein with a PEI–glutathione conjugate when they were mixed together. This complex was found to be efficiently delivered into living cells to exert biological activity of the GST-fused protein.

EXPERIMENTAL PROCEDURES

Materials—Polyethylenimines (PEIs, Epomin™ SP series: manufactured by Nippon Shokubai, Osaka) with average molecular masses of 600 (PEI600) and 1800 (PEI1800) were donated by Nippon Shokubai or purchased from Wako Chemical (Osaka, Japan). 1,3-Butadiene monoepoxide was purchased from TCI (Tokyo, Japan). Aldrithiol (2,2′-dithiodipyridine), γ-thiobutyrolactone and mouse anti-MAP kinase activated (diphosphorylated ERK-1&2) clone MAPK-YT mouse ascites fluid (anti-pERK antibody) were purchased from Sigma (St Louis, MO, USA). Rabbit anti-ERK 1/2 antibody was purchased from Promega (Madison, WI, USA). Goat anti-GST antibody was purchased from GE Healthcare (Buckinghamshire, England). A MAP kinase assay kit [containing myelin basic protein (MBP) and anti-pMBP monoclonal antibody] was purchased from Upstate (Lake Placid, NY, USA).

Synthesis of Hydroxybutenyl PEI600 (GX4)—Hydroxybutenyl PEI600 was synthesized by mixing PEI600 with three times molar excess 1,3-butadiene monoepoxide. Briefly, PEI600 (5.0 g, 8.3 mmol) dissolved in 20 ml of tetrahydrofuran (THF) was mixed with 1,3-butadiene monoepoxide (1.75 g, 25.0 mmol) and then incubated for 5 h at 50°C. After removal of THF by evaporation, the residual oil, hydroxybutenyl PEI600, was designated as GX4 and stored in the dark at room temperature until use. The number of hydroxybutenyl moiety in a molecule of GX4 was found to be ranged from 0 to 3 (the average number was about one) when it was determined by MALDI-TOF mass spectrometry with a Perspective Voyager-DE PRO mass spectrometer.

Preparation of PEI–Glutathione Conjugates—PEI600–glutathione conjugates, in which various amounts of glutathione were conjugated to PEI600 through disulfide bond, were prepared in reactions employing the following molar ratios of reagents, PEI600 : γ-thiobutyrolactone : aldrithiol : reduced form of glutathione to be 1 : 1 : 1.1 : 0.9 (after the approximate molar ratio of PEI600 to reduced form of glutathione used, the product was designated as PEI600–glutathione$_1$), 1 : 2 : 2.2 : 1.8 (designated as PEI600–glutathione$_2$) and 1 : 3 : 3.3 : 2.7 (designated as PEI600–glutathione$_3$), respectively. PEI600–glutathione$_3$, for example, was prepared as follows. To a solution of 1.2 g of PEI600 (2.0 mmol) in 5 ml of THF were added 1.45 g of aldrithiol (6.6 mmol) and 0.61 g of γ-thiobutyrolactone (6.0 mmol) with stirring, and the mixture was warmed at 50°C for 2 h. During the reaction, the mixture separated into two layers. After removal of THF by evaporation, the residual gelatin-like gel was dissolved in Milli-Q water to 13.3 ml (150 mM based on the PEI moiety) and kept for 1 h at room temperature to allow the reaction of free SH groups with aldrithiol to be complete. At this stage, small aliquot was withdrawn to determine the amount of reacted aldrithiol by UV spectroscopy at 343 nm assuming a molar extinction coefficient of 2-thiopyridone to be $\varepsilon_{343} = 8080/M/cm$. The solution was then treated with the reduced form of glutathione (830 mg, 2.7 mmol) at room temperature for overnight to give PEI600–glutathione$_3$, which was stored at room temperature until use. Similarly, PEI600–glutathione$_n$ ($n = 1$ and 2), PEI1800–glutathione$_n$ ($n = 1$, 2 and 3) and GX4–glutathione$_3$ were also prepared.

Cell Culture, Protein Transduction and Plasmid DNA Transfection—Cells were grown in monolayer cultures in RPMI1640 medium (Sigma) (CHO and HelaS3 cells) or Dulbecco's modified Eagle's medium (DMEM, Sigma) (Balb/c 3T3 and Cos7 cells) supplemented with 10% fetal bovine serum (FBS) and 70 μg/ml of kanamycin at 37°C in a humidified 5% CO_2 atmosphere. For protein transduction experiments, about 80% confluent cells were washed once with serum-free medium, and then a sample was added under serum-free conditions. For plasmid DNA transfection experiments, the medium of cell culture was changed to Opti-MEM (Invitrogen, Carlsbad, CA, USA), and plasmid DNA was transfected with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Plasmid Constructs—A GST-enhanced green fluorescent protein (eGFP)-fused protein (GST–eGFP) expression vector was constructed by inserting eGFP from pEGFP-N1 (Clontech, Mountain View, CA, USA) into pGEX-4T-3 vector (GE Healthcare) at the XmaI and NotI sites. A GST-constitutively active mitogen-activated protein kinase kinase 1 (caMEK1, S216E/S222E, Δ32–51)-fused protein (GST-caMEK1) expression vector was constructed by inserting caMEK1 from pFC-MEK1 (Stratagene, La Jolla, CA, USA) into pGEX-6P-1 vector (GE Healthcare) at the BamHI sites. A GST and mitogen-activated protein kinase 1 (ERK1)-fused protein (GST-ERK1) expression plasmid DNA was kindly provided by Prof. Y. Gotoh (Tokyo University).

Expression and Purification of Recombinant Proteins—All recombinant proteins were produced in *E. coli* BL21(DE3)plysS (Novagen, Madison, WI, USA). Transformed *E. coli* cells were cultured in Luria–Bertani medium containing 200 μg/ml of ampicillin at 37°C. When the optical density of the medium at 600 nm reached ~0.8, 0.5 mM of isopropyl 1-thio-β-D-galactopyranoside was added and the cells were cultured for another 15 h at 25°C. Harvested *E. coli* cells were then lysed by repeating a freeze/thaw/sonication cycle twice. Resulting lysates were centrifuged to remove insoluble debris, and recombinant proteins were purified from supernatants by a Glutathione Sepharose 4B column (GE Healthcare). Briefly, the cell lysate was applied to the column, non-specifically bound proteins were washed out with PBS containing 0.1% Tween-20, and then

specifically bound GST-fused proteins were eluted with 50 mM Tris–HCl (pH 8.0) containing 10 mM of the reduced form of glutathione. Recovered GST-fused proteins were dialysed against PBS to remove glutathione. Because the purity of affinity-purified GST-caMEK1 was not so high, it was further fractionated by gel filtration on Sephacryl S-200 HR (GE Healthcare, 1.5 × 30 cm) in 20 mM HEPES buffer (pH 7.3) and 150 mM NaCl. Recovered GST-caMEK1 was subsequently purified by ion-exchange HPLC by use of a RESOURCE-Q column (GE Healthcare) eluted under a linear gradient of NaCl from 0 to 0.5 M in 20 mM HEPES buffer (pH 7.3) for 60 min at a flow rate of 0.8 ml/min. Purified GST-caMEK1 was appropriately concentrated by an Amicon Ultra centrifugal filter (10 000 MWCO) (Millipore, Bedford, MA, USA).

Western Blot Analysis—Cell lysates were prepared using lysis buffer containing 40 mM Tris–HCl, pH 7.4, 1% TritonX-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 10% glycerol, 0.1% SDS, 50 mM NaF and 1 mM Na_3VO_4, and were clarified by centrifugation. The cell extracts were subjected to SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with anti-GST antibody, anti-pERK antibody, anti-ERK 1/2 antibody or anti-pMBP antibody, followed by the application of alkaline phosphatase-conjugated anti-goat IgG antibody (EY laboratories, San Mateo, CA, USA), horseradish peroxidase-conjugated anti-mouse IgG antibody or horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology Inc., Beverly, MA, USA). Positive signals were visualized using a CDP-Star Chemiluminescent substrate for alkaline phosphatase (New England BioLabs, Ipswich, MA, USA) or a Western Lightning Chemiluminescence Reagent Plus for horseradish peroxidase (Perkin Elmer LAS, Boston, MA, USA).

Fluorescent Imaging—Intracellular delivery of GST–eGFP assisted by PEI–glutathione conjugates was observed by using a confocal laser-scanning microscope (model LSM 510; Carl Zeiss, Jena, Germany). In order to confirm protein transduction, the medium of exponentially growing cells on a glass-based dish was changed to serum-free medium, and GST–eGFP was added with or without PEI–glutathione conjugate. After incubation for 2 h at 37°C, the cells were incubated with the medium containing 20 mM of DTT for 10 min at 37°C to remove the protein on the cellular surface, washed twice with PBS and directly observed.

Immunofluorescence Staining—Cells were fixed with 4% paraformaldehyde for 1 h at room temperature and then permeabilized with 70% ethanol at −20°C. Immunofluorescence staining for phosphorylated ERK was performed by treatment of cells with anti-pERK antibody followed by treatment with Alexa Fluor

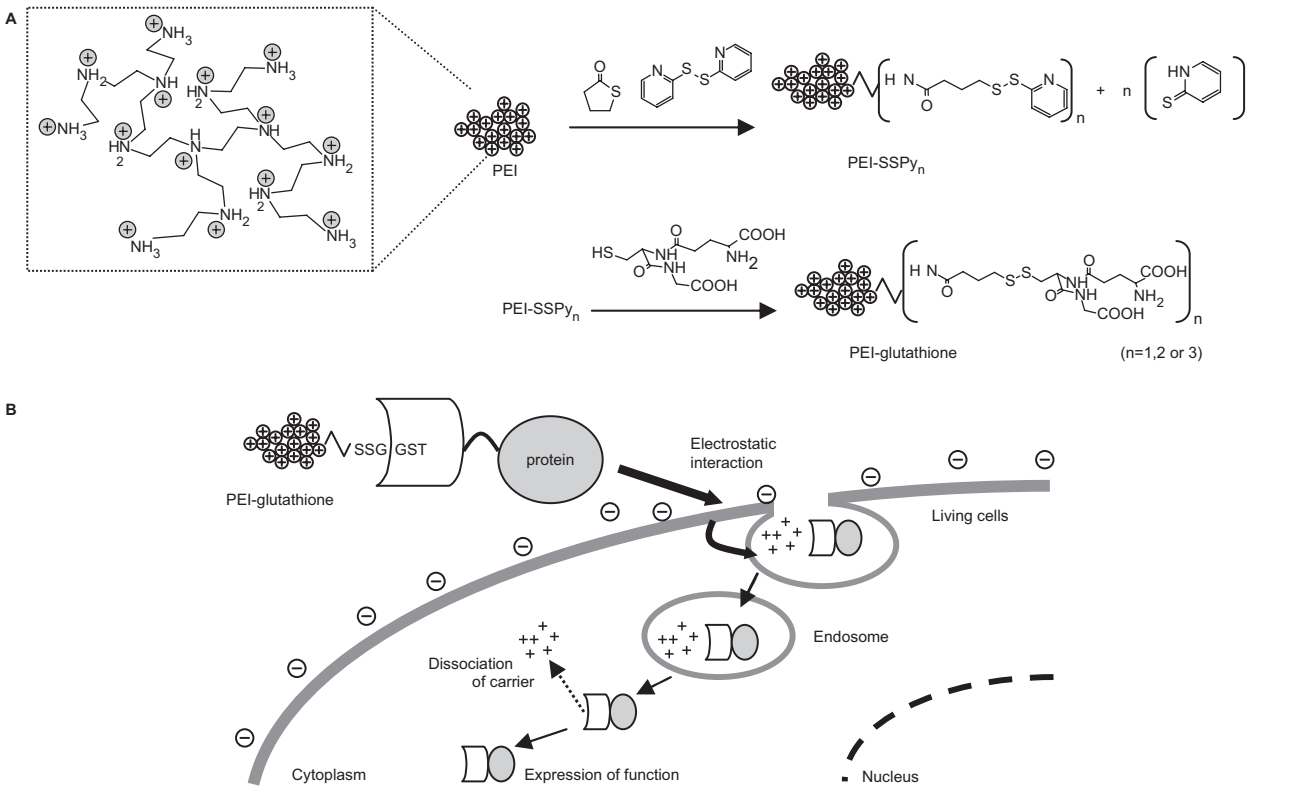

Fig. 1. **Schematic diagrams of the preparation of PEI–glutathione and the transduction of GST-fused protein assisted by PEI–glutathione.** PEI–glutathione conjugates were prepared in two-stage reaction procedure (A). A GST-fused protein cationized with PEI–glutathione conjugate by affinity complex formation was adsorbed on a cellular surface by electrostatic interaction and then internalized into the cell via endocytosis. When the complex reaches the cytoplasm, the carrier of PEI will rapidly dissociate from the GST-fused protein because exposed disulfide bonds between PEI and glutathione are cleavable under a cytosolic reducing environment (B).

488 goat anti-mouse IgG antibody (Invitrogen). Nuclei were stained with Hoechst 33258 (Dojin Laboratories, Kumamoto, Japan).

RESULTS AND DISCUSSION

Characterization of PEI–Glutathione Conjugates—As stated in the introduction, proteins possessing a cationic net-charge show efficient and non-cytotoxic internalization into cells through adsorptive-mediated endocytosis. Therefore, this principle is a notable point for the development of protein transduction technology (*1*). A chemically synthesized molecule of PEI is a one of the most suitable materials for protein cationization. PEI is a polycationic polyamine with a branched backbone of two carbons followed by one potentially protonated nitrogen atom in every molecular mass unit of 43 (*14*). This value (1/43) is 3.7-fold and 3.0-fold higher than those of poly-Arg (1/157) and poly-Lys (1/129), respectively. We hence employed PEI as a cationic carrier for cationization of GST-fused proteins.

To conduct a PEI cationization of GST-fused protein, we simply synthesized PEI–glutathione conjugates which were able to form complexes with GST-fused protein *via* the affinity between glutathione and GST. As shown in Fig. 1A, PEI–glutathione conjugates were prepared in two-stage reaction procedure. In the first stage, PEI600 was reacted with about one, two or three times molar excess γ-thiobutyrolactone in the presence of aldrithiol to obtain PEI600 derivatives modified with approximately one, two or three 4-(2-pyridyldithio)butyroyl groups per one PEI600 molecule (PEI600-SSPy$_n$, $n = 1.2$ or 3 in Fig. 1A), respectively. However, the yield of 2-thiopyridone, a byproduct of the reaction, determined by UV spectrometry, was found to be about 60% in every case (56, 58 or 57% for $n = 1$, 2 or 3, respectively). Since almost all γ-thiobutyrolactone was considered to be reacted with PEI to produce SH group attached to PEI, these results suggest that only about 60% of the SH group was protected with aldrithiol to give the same amount of 2-thiopyridone as a byproduct but the rest (about 40%) was air-oxidized to give disulfide bonded oligomerized PEI600 derivatives (*vide infra*). In the second stage of the reaction procedure, 2-thiopyridyl moieties were replaced with glutathione moieties. SDS–PAGE analysis of the final reaction mixtures showed the presence of some oligomerized PEI derivatives at least up to trimer (data not shown) in all cases. Thus, the all reaction mixtures contained some oligomerized PEI600 although we simply designated them as PEI600–glutathione$_n$ ($n = 1$, 2 and 3), where each n reflected the molar ratio of γ-thiobutyrolactone (proportional to the amount of glutathione conjugated) to PEI600. Because PEI600 itself was a mixture of molecules of average molecular mass of 600, the PEI600–glutathione conjugates were more widely dispersed complex mixtures. But we could roughly approximate that PEI600–glutathione$_n$ ($n = 1$, 2 or 3) was a mixture of monomer, dimer and trimer of PEI600, and number of glutathione attached to a partly oligomerized PEI600 molecule was about 'n' in average, respectively. Similarly, PEI1800–glutathione conjugates, PEI1800–glutathione$_n$ ($n = 1$, 2 and 3) and a hydroxybutenylated PEI600–glutathione conjugate, GX4–glutathione$_n$ ($n = 3$), were also considered to be partly oligomerized PEI mixtures conjugated with approximately n's glutathione moieties, respectively. Because purification of any single PEI–glutathione conjugate form the complex mixture described above was extremely difficult, the reaction mixtures were directly examined about the GST-fused protein transduction ability into living cells. In these PEI–glutathione conjugates, glutathione was bonded through disulfide bonds with primary or secondary amines on the molecular surface of PEI, and the coupling sites would be exposed to a solvent. Therefore, these disulfide bonds are expected to be sensitive to reduction in cytosolic reducing environments (Fig. 1B) (*17*). This property should be suitable for a GST-protein to express its function after transduction into cells.

Quantitative Analysis of GST Transduction into CHO Cells—To investigate the transduction efficiency of a

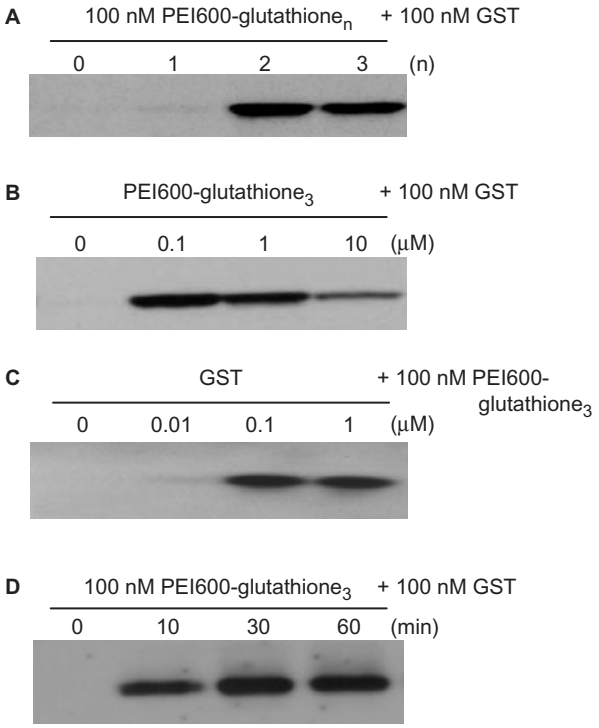

Fig. 2. **Transduction of GST into cells by PEI–glutathione conjugates.** CHO cells were incubated with PEI–glutathione conjugates and GST before total cell lysates were subjected to SDS–PAGE and transferred onto nitrocellulose membrane, which was analysed by western blot analysis with an anti-GST antibody. The total GST, both bound to the cellular surface and from intracellular uptake, was detected. Conjugation ratios of PEI600 to glutathione suitable for protein transduction were examined by incubation of cells with 100 nM of each PEI600–glutathione$_n$ (n = 1, 2 or 3) and 100 nM of GST for 1 h (A). Various mixing ratios of GST (100 nM) and PEI600–glutathione$_3$ (0–10 μM) were compared by incubation with cells for 1 h (B). Various mixing ratios of GST (0–1 μM) and PEI600–glutathione$_3$ (100 nM) were compared by incubation with cells for 1 h (C). Time course of cellular uptake of an equimolar (100 nM) mixture of GST and PEI600–glutathione$_3$ was examined after incubation for indicated periods (D).

GST-fused protein assisted by PEI600–glutathione conjugates, GST itself was transduced and the total amount of GST in cell lysate was assessed by western blotting. Since the major intracellular uptake route of a PEI-cationized protein is thought to be an adsorptive-mediated endocytosis, this amount would correlate with the protein transduction efficiency. Firstly, 100 nM of PEI600–glutathione$_n$ ($n = 1$, 2 or 3) was added with 100 nM of GST to the culture medium of CHO cells. The concentration of each PEI600–glutathione$_n$ was expressed as that assuming PEI not to be oligomerized. After incubation for 1 h, GST was detected in total the cell lysate when GST was mixed with multivalent ($n = 2$, 3) conjugates. However, a monovalent ($n = 1$) conjugate and PEI600 itself virtually failed in promoting GST transduction (Fig. 2A). These results suggest that multivalent conjugates, but not a monovalent one, can form stable affinity complexes with GST under experimental conditions. We next explored the optimal mixing ratio of GST and PEI600–glutathione$_3$ for protein transduction. As shown in Fig. 2B and C, an equimolar mixture of GST and PEI600–glutathione$_3$ showed efficient protein transduction but 10 times excess PEI600–glutathione$_3$ decreased the protein transduction efficiency, while 10 times excess GST showed almost the same efficiency. Therefore, an equimolar mixture of GST and PEI–glutathione$_3$ was used for subsequent experiments. To confirm the time-dependent protein transduction, an equimolar mixture of GST and PEI600–glutathione$_3$ was added to the culture medium of CHO cells. As shown in Fig. 2D, GST was found to be adsorbed to and/or internalized into cells within 10 min by forming an affinity complex with PEI600–glutathione$_3$, and the amount gradually increased over a period of 1 h.

Imaging of Intracellular Delivery of GST–eGFP by PEI–Glutathione Conjugates—To visualize the protein transduction assisted by PEI–glutathiones, GST–eGFP was selected as a model protein to be transduced. In fluorescent microscopic observation of adsorptive-mediated endocytosis in living cells, it is difficult to

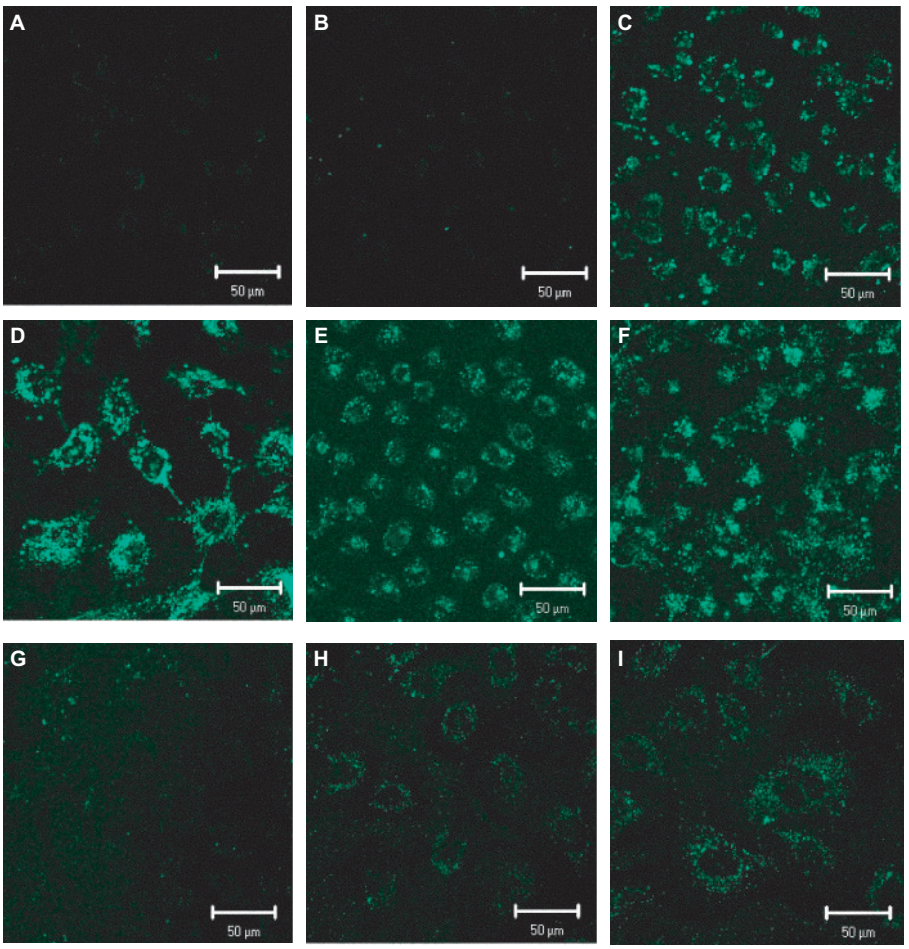

Fig. 3. **Intracellular delivery of GST–eGFP with PEI–glutathione conjugates.** Intracellular delivery of GST–eGFP into CHO cells was analysed by incubating cells with 500 nM GST–eGFP alone (A), with 500 nM eGFP (GST-null) and 500 nM PEI600–glutathione$_3$ (B) or with 500 nM GST–eGFP and 500 nM PEI600–glutathione$_3$ (C) for 2 h in the absence of FBS. Balb/c 3T3 (D), HeLa S3 (E) and Cos7 (F) cells were treated with 500 nM GST–eGFP and 500 nM PEI600–glutathione$_3$ for 2 h in the absence of FBS. Balb/c 3T3 cells were treated with 500 nM GST–eGFP and 500 nM PEI1800–glutathione$_1$ (G), 500 nM PEI1800–glutathione$_2$ (H) or 500 nM PEI1800–glutathione$_3$ (I) for 2 h. These cells were then incubated with a medium containing 20 mM DTT for 10 min, washed with PBS and examined under a confocal laser-scanning microscope. The scale bars are equivalent to 50 μm.

distinguish strong fluorescence from eGFP molecules adsorbed to the cellular surface and relatively weak one from those in intracellular uptake compartments. Therefore, we treated cells with 20 mM of DTT for 10 min at 37°C just before microscopic observations to wash out GST–eGFP on the cellular surface. This treatment condition was confirmed to be sufficient for cleaving disulfide bonds in PEI–glutathione conjugates and dissociating the affinity complex, but was not cytotoxic. When CHO cells were treated with an equimolar mixture of GST–eGFP and PEI600–glutathione$_3$, fluorescence from discrete intracellular endosomes was observed (Fig. 3C), whereas no fluorescence was observed in cells treated with GST–eGFP alone (Fig. 3A) or eGFP (GST-null) and PEI600–glutathione$_3$ mixture (Fig. 3B). Successful protein transduction of GST–eGFP was also observed in Balb/c 3T3 (Fig. 3D), Hela S3 (Fig. 3E) and Cos7 (Fig. 3F) cells. Namiki *et al.* (*20*) reported that fluorescent dye-labelled GST itself penetrated into cells. However, we did not observe cellular uptake of either GST or GST–eGFP. As GST might have only a little ability to enter cells, a PEI-cationization strategy of a GST-fused protein *via* binding to a glutathione moiety was demonstrated to provide a powerful pathway for protein transduction.

We previously observed that the net positive charge of proteins correlated well with their efficiency for protein transduction (*12–14*). In this point of view, PEI–glutathione conjugates with PEIs of higher molecular mass that possess more protonatable amines were thought to be more suitable for efficient protein transduction of highly acidic GST-fused proteins. According to this hypothesis, we prepared glutathione conjugates of more cationic PEI1800 to enhance protein transduction efficiency. However, PEI1800–glutathione$_n$ ($n = 1$, 2 and 3) showed less efficient protein transduction (Fig. 3G–I) than PEI600–glutathione$_3$ did (Fig. 3D). Less efficiency in the protein transduction of PEI1800–glutathiones suggests that binding affinity between GST–eGFP and PEI1800–glutathione$_n$ is not sufficient to form a stable affinity complex, presumably due to the lower density of glutathione moieties on the molecular surface of PEI1800 than that on PEI600. In other words, more clustered glutathiones on the surface of PEI600 would contribute to the formation of a more stable affinity complex with GST than the less clustered glutathiones on PEI1800 in similar multivalent ($n = 2$ and 3) conjugates. Since the reported value for apparent dissociation constant (K_D) of GST and glutathione reported (0.2–0.6 mM) (*21*) is comparable to the concentration of GST or its fused protein (100–500 nM) used in our experiments, a possible explanation of the present results is that clustering of glutathione moieties increases its effective concentration to decrease the apparent K_D by an entropic effect and this effect is more pronounced in PEI600 than in PEI1800 because of the difference in density of glutathione moiety on the conjugate.

Enhanced Endosomal Escape by Modified PEI–Glutathione Conjugates—As shown in Fig. 3, PEI-cationized GST-fused proteins appeared to be delivered into cells by adsorptive-mediated endocytosis. After being taken into the cells by endocytosis, GST-fused proteins have to escape from endocytic vesicles for efficient expression of biological activity in cells. Since endosomal escape is often observed as an inefficient process, endosomal escape has been pointed out as one of the most important subjects for transduction of genes, proteins and drugs into cells. Some approaches to destabilize the endosome, including the use of a TAT-HA fusogenic peptide (*22*), photo-acceleration (*23*), the use of lysosomotrophic agents (*24*) and the use of a pH-sensitive polymer (*25*), have been reported. A study on PEI-mediated DNA transfection has also suggested that PEIs possess the ability of endosomal escape as a consequence of high

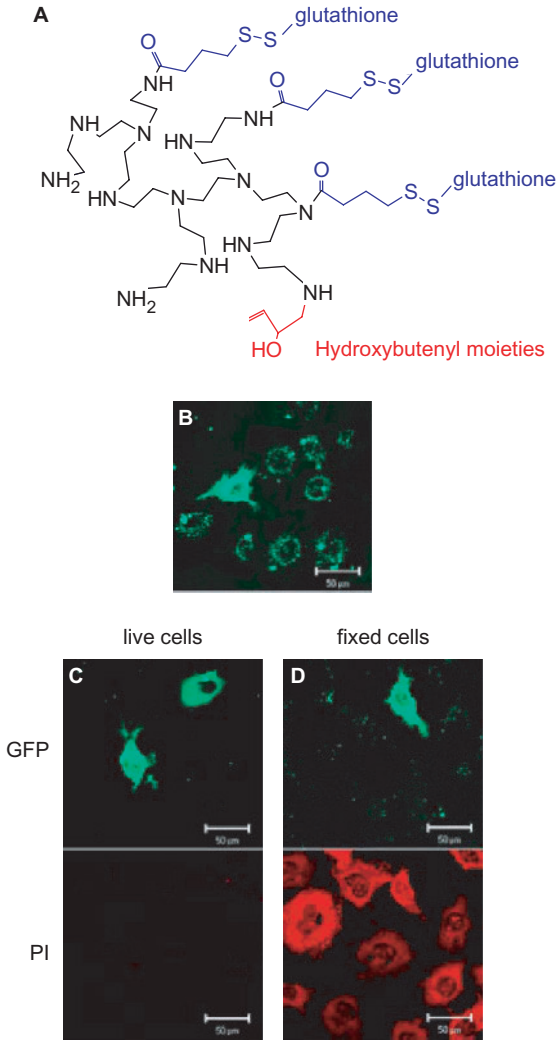

Fig. 4. **Enhanced endosomal escape by chemically modified PEI–glutathione conjugate.** Predicted structure of GX4–glutathione$_3$ conjugate (A). Balb/c 3T3 cells were incubated with 500 nM GST–eGFP and 500 nM GX4–glutathione$_3$ in the absence of FBS for 2 h. Fluorescent images were obtained after removal of the cellular surface fraction of GST–eGFP by incubation with 20 mM DTT for 10 min (B). The cells were incubated with 500 nM GST–eGFP and 500 nM GX4–glutathione$_3$ as (B), and then with 1 μg/ml PI for 20 min directly (C, live cells) or after fixation with 4% paraformaldehyde and permeabilization with 1% TritonX-100 (D, fixed cells). These cells were then washed with PBS and examined under a confocal laser-scanning microscope. The scale bars are equivalent to 50 μm.

buffering capacity (*26*). Although the PEI–glutathione conjugates used in this study may have this so-called capacity proton sponge effect, the effect would not be significant because PEI600 is much smaller than the PEIs (Mr. 70,000–800,000) used for DNA transfection (*27*).

To develop low-molecular mass PEIs endowed with enhanced endosomal escape ability, a series of chemically modified PEI600 derivatives were conjugated with glutathione, and then their protein transduction and endosomal escape ability were evaluated by fluorescent images of GST–eGFP. In this screening, we found that GX4–glutathione$_3$, a PEI600–glutathione$_3$ derivative possessing a hydroxybutenyl moiety (Fig. 4A), showed excellent ability for this purpose. As shown in Fig. 4B, about 10% of cells showed intracellularly diffused fluorescence distribution when GST–GFP was transduced by GX4–glutathione$_3$. Furthermore, this reagent was confirmed not to kill cells along with endosomal escape, because these fluorescence-diffused cells were not stained with propidium iodide (PI) (Fig. 4C), while these cells were stained after fixation as usual (Fig. 4D).

To test the usefulness of PEI–glutathione-mediated transduction of GST-fused proteins for controlling cellular function, we transduced a biologically functional GST-fused protein into living cells. As a model protein, we employed a constitutively active form of MEK1 (caMEK1, S216E/S222E, Δ32–51). The biological activity of GST-caMEK1 was confirmed by an *in vitro* kinase assay in which the kinase cascade is reconstituted by addition of GST-ERK1, adenosine triphosphate (ATP) and a substrate of MBP (Fig. 5A). After transduction of GST-caMEK1 by PEI600–glutathione$_3$ at 500 nM for 2 h into quiescent cells, non-phosphorylated endogenous ERK proteins (ERK1 and ERK2) were found to be partly phosphorylated (Fig. 5B, pERK). Furthermore GX4–glutathione$_3$ showed more induction of ERK-phosphorylation than PEI600–glutathione$_3$ did, but its level was not as high as that with 10% FBS stimulation. The induction was also confirmed by immunofluorescence staining of cells (Fig. 5C). Although ERK-phosphorylation was hardly observed under 0.5% serum condition (control of Fig. 5C), 10% FBS induced ERK-phosphorylation more or less in all cells

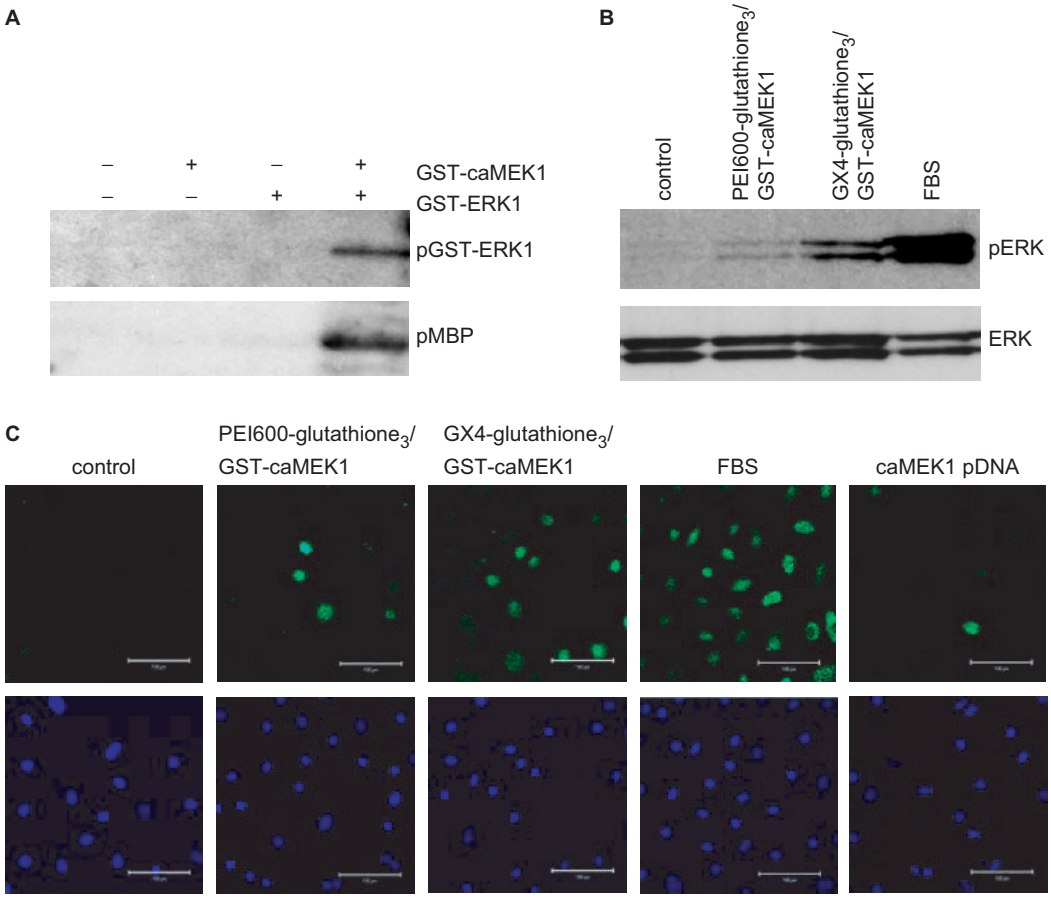

Fig. 5. **Transduction of GST-caMEK1.** The enzymatic activity of GST-caMEK1 was confirmed using an *in vitro* reconstituted MAP kinase assay kit. The phosphorylation of GST-ERK1 and MBP was examined by western blot analysis using an anti-pERK or anti-pMBP antibody (A). Activation of endogenous ERK by exogenous GST-caMEK1 was demonstrated by the use of Balb/c 3T3 cells (B and C). Cells had been starved for 24 h in a medium containing 0.5% FBS, and then incubated with 500 nM protein samples or 10% FBS for 2 h, or with plasmid DNA/lipofectamine 2000 for 24 h. The phosphorylated-ERK protein and total ERK protein in each cell lysate were examined by western blot analysis using an anti-pERK antibody or anti-ERK antibody (B) or phosphorylated-ERK in cells was visualized using an anti-pERK antibody and Alexa Fluor 488 anti-mouse IgG antibody (C, upper panels). Nuclei were also visualized by staining with Hoechst 33258 (C, lower panels). The scale bars are equivalent to 100 μm.

(FBS of Fig. 5C). Transfection of plasmid DNA encoding caMEK1 for 24 h induced ERK-phosphorylation in about 10% of cells (caMEK1 pDNA of Fig. 5C). Interestingly, when caMEK1 was delivered into cells with PEI600–glutathione$_3$ or GX4–glutathione$_3$, ERK-phosphorylation was more efficiently induced in cells after 2 h incubation. The ratio of cells showing the ERK-phosphorylation level comparable to that observed in 10% FBS-treated cells was about 20% for PEI600–glutathione$_3$/GST-caMEK1 and 60% for GX4–glutathione$_3$/GST-caMEK1, respectively. These results suggest that the protein transduction method is sometimes more useful than the plasmid DNA transfection method, and that the superiority of GX4–glutathione$_3$ to PEI600–glutathione$_3$ might be responsible to its enhanced ability of endosomal escape.

In conclusion, we developed a novel protein transduction tool for GST-fused proteins by use of a polycationic PEI and its derivatives. In their preparations, glutathiones were conjugated *via* cleavable disulfide bonds to PEIs, and sufficient clustered glutathione conjugation was found to be necessary for the formation of a stable complex with GST-fused proteins. So far, optimized PEI–glutathione conjugate for protein transduction was characterized by a partly oligomerized mixture of PEI with average molecular masses of 600 (PEI600) modified with about three glutathiones. Furthermore, hydroxybutenylated PEI showed enhanced endosomal escape ability as demonstrated by diffused intracellular fluorescence of GST–eGFP and up-regulation of phosphorylation of an endogenous target protein with GST-caMEK1. This protein transduction experiment can be easily performed by just adding a mixture of a 1 : 1 (mol/mol) ratio of GST-fused protein and a PEI–glutathione conjugate to the culture medium of cells. Since GST-fused protein expression and purification systems have been widely used, there may be large numbers of GST-fused proteins in refrigerators or freezers of many laboratories. There are many evidences that GST-fused proteins could exhibit biological activity in living cells because as shown in numbers of microinjection experiments in which protein functions have been analysed by using GST-fused proteins (*28–31*). Of course there is a limitation of GST-tag on *in vivo* usage because of size effect, possible immunogenicity, etc., but the development of a protein transduction tool specific for GST-fused proteins may greatly contribute to the promotion of protein transduction experiments by effective utilization of large numbers of resting samples.

We thank Dr Y. Gotoh for her kind gift of GST-ERK1 expression plasmid. This work was performed as a research and development project of the Industrial Science and Technology Program supported by the New Energy and Industrial Technology Development Organization (NEDO). This work was partly supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (17360399 and 19206085 to H.Y.).

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