Denatured and Reversibly Cationized p53 Readily Enters Cells and Simultaneously Folds to the Functional Protein in the Cells†

Hitoshi Murata,‡ Masakiyo Sakaguchi,§ Junichiro Futami,‡,||,⊥ Midori Kitazoe,‡ Takashi Maeda,‡ Hideki Doura,‡ Megumi Kosaka,‡ Hiroko Tada,‡ Masaharu Seno,‡,# Nam-ho Huh,§ and Hidenori Yamada*,‡,⊥

Department of Bioscience and Biotechnology, Faculty of Engineering, Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan, Department of Cell Biology, Graduate School of Medicine and Dentistry, Okayama University, Okayama 700-8558, Japan, Nippon Shokubai Co., Ltd., 5-8 Nishi Otabi-cho, Suita, Osaka 564-8512, Japan, Engineering Innovation Center, Okayama University, Okayama 700-853, Japan, and Research Center for Biomedical Engineering, Okayama University, Okayama 700-8530, Japan

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ABSTRACT: Cationization is a powerful strategy for internalizing a protein into living cells. On the other hand, a reversibly cationized denatured protein through disulfide bonds is not only soluble in water but also able to fold to the native conformation in vitro. When these advantages in cationization were combined, we developed a novel method to deliver a denatured protein into cells and simultaneously let it fold to express its function within cells. This "in-cell folding" method enhances the utility of recombinant proteins expressed in *Escherichia coli* as inclusion bodies; that is, the recombinant proteins in inclusion bodies are solubilized by reversible cationization through cysteine residues by disulfide bonds with aminopropyl methanethiosulfonate or pyridyldithiopropionylpolyethylenimine and then incubated with cells without an in vitro folding procedure. As a model protein, we investigated human tumor-suppressor p53. Treatment of p53-null Saos-2 cells with reversibly cationized p53 revealed that all events examined as indications of the activation of p53 in cells, such as reduction of disulfide bonds followed by tetramer formation, localization into the nucleus, induction of p53 target genes, and induction of apoptosis of cells, occurred. These results suggest that reversible cationization of a denatured protein through cysteine residues is an alternative method for delivery of a functional protein into cells. This method would be very useful when a native folded protein is not readily available.

It was proposed more than a decade ago that a cationization method can be used for delivery of a protein into cells by adsorption-mediated endocytosis (*1*). To deliver a protein into living cells and let it function within cells, we have developed a method in which a protein is modified with a cationic amine such as ethylenediamine or polyethylenimine (PEI)[1] (*2*−*5*). The method exploits the electrostatic interaction of a cationized protein with the negatively charged cell membrane. From PEI cationization, a protein with a native structure and function was efficiently delivered into cells. However, purification of a large amount of a protein and preservation of its activity during the cationization procedures are sometimes difficult.

The technique of recombinant-protein expression in *Escherichia coli* is widely used to obtain a large amount of protein. However, overexpression of recombinant proteins in *E. coli* often results in the accumulation of insoluble inclusion bodies. Because most denatured proteins are hardly soluble in water, it is difficult to purify them by conventional chromatographic procedures. We have reported that introduction of an efficient positive charge by S-alkylation can solubilize reduced and denatured proteins in water (*6*). We have also developed a method for solubilization of a reduced and denatured protein by reversible modification of cysteine residues with a cationic charge through disulfide bonds. The latter solubilized denatured protein could be not only purified but also further folded to the native conformation in vitro (*7*−*11*).

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* To whom correspondence should be addressed. Telephone: +81-86-251-8215. Fax: +81-86-251-8265. E-mail: yamadah@cc.okayama-u.ac.jp.

‡ Graduate School of Natural Science and Technology, Okayama University.

§ Graduate School of Medicine and Dentistry, Okayama University.

|| Nippon Shokubai Co., Ltd.

⊥ Engineering Innovation Center, Okayama University.

Research Center for Biomedical Engineering, Okayama University.

[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(2-aminoethylether)-tetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4NQO, 4-nitroquinoline 1-oxide; PBS, phosphate-buffered saline; PEI, polyethylenimine; PEI600, PEI with an average molecular mass of 600; p21/waf1, wild-type p53-activated fragment 1; PTD, protein transduction domain; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SV40, simian virus 40; SVLT, SV40 large-T antigen; Tris, tris(hydroxymethyl)aminomethane; TRITC, tetramethylrhodamine B isothiocyanate.

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Cells should have inherent folding machinery for denatured proteins. Thus, using reversible cationization, we developed an "in-cell folding" method for a denatured protein, a method to deliver an unfolded protein into cells and let it fold within cells. Using this method, the human tumor-suppressor p53 expressed in *E. coli* as inclusion bodies was successfully delivered into and folded to the active form within p53-null Saos-2 cells.

MATERIALS AND METHODS

Materials. PEI with an average molecular mass of 600 (PEI600) was purchased from Wako Chemical (Osaka, Japan). *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Pierce (Rockford, IL). An antibody that recognizes the native conformation of wild-type human p53 (anti-native p53 antibody Ab-5) was purchased from Calbiochem (La Jolla, CA). For Western blot analysis, mouse anti-human p53 antibody (anti-p53 antibody Bp53-12, a product of Santa Cruz Biotechnology, Inc.), mouse anti-human tubulin antibody (anti-tubulin antibody), and mouse anti-human β-actin antibody (anti-β-actin antibody) were purchased from Sigma (St. Louis, MO). Mouse anti-p21/wild-type p53-activated fragment 1 antibody clone 6B6 (anti-p21/waf1 antibody 6B6) was purchased from Becton Dickinson (San Jose, CA). For immunofluorescence staining of cells, mouse anti-p21/wild-type p53-activated fragment 1 antibody clone 187 (anti-p21/waf1 antibody 187) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Human osteogenic sarcoma-derived Saos-2 cells were obtained from American Type Culture Collection ATCC (Rockville, MD). Normal human fibroblast OUMS-24 cells were those as described elsewhere (*12*). A plasmid encoding human wild-type full-length p53 (pCB6 + p53Pro) was provided by Dr. Karen Vousden.

Synthesis of 3-Aminopropyl Methanethiosulfonate Hydrobromide (APS-Sulfonate) and Pyridyldithiopropionylpolyethylenimine (PEI600-SPDP). APS-sulfonate was synthesized according to the method for the synthesis of 3-(trimethylammonio)propyl methanethiosulfonate bromide [CH_3SO_2-$SCH_2CH_2CH_2N^+(CH_3)_3 \cdot Br^-$] (TAPS-sulfonate) (*7*), except for the use of 3-bromopropylamine hydrobromide instead of (3-bromopropyl)trimethylammonium bromide. APS-sulfonate was obtained as white crystals from ethanol ether in about 80% yield and melted at 121−124 °C. PEI600-SPDP solution was prepared by just mixing a PEI600 solution (200 mg/mL, 0.33 M, pH 8 adjusted by HCl) with SPDP dissolved in ethanol (0.02 M, at a molar ratio of 5:1 PEI600/SPDP). The solution was incubated at room temperature for 20 min and then stored at 4 °C until use.

Protein Expression and Isolation of Inclusion Bodies. The expression plasmid for p53, pBO429, was constructed by the following digestion and ligation steps. The coding region for human wild-type full-length p53 was excised from pCB6 + p53Pro, at the *Nco*I (by partial digestion) and *Bam*HI sites, and the fragment obtained was inserted between the *Nco*I and *Bam*HI sites of an overexpression vector, pET14b (Novagen, WI). Recombinant p53 was expressed in *E. coli* BL21(DE3) (Novagen) harboring pBO429. For overexpression of p53, the cells were cultured at 37 °C in terrific broth containing 200 μg/mL of ampicillin until A_{600} reached 0.8 and were then treated with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and cultured for another 3 h. The cells were then pelleted and lysed by repeating a freeze/thaw/sonication cycle twice. The isolation of inclusion bodies was performed according to the published procedure (*13*, *14*).

Reversible Cationization of Protein. The recombinant p53 in the inclusion bodies was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer at pH 8.6 containing 6 M guanidine-HCl and reduced with 3 mM dithiothreitol (DTT) at 37 °C for 1 h under N_2 atmosphere. Sulfhydryl (SH) groups of p53 were then modified with APS-sulfonate or PEI600-SPDP to give AP-SS-p53 or PEI600-SS-p53, respectively, which were denatured and reversibly cationized p53 proteins containing cationic groups through disulfide (SS) bonds (Figure 1A). Namely, the reaction was initiated by adding 9 mM APS-sulfonate or PEI600-SPDP to the above denatured and reduced p53 solution, and the solution was incubated at 37 °C for 1 h. In the reaction with a relatively large size of PEI600-SPDP, the modification of cysteine residues in p53 was incomplete, probably because of steric hindrance (see below), and the remaining SH groups catalyzed protein polymerization by SH−SS interchange during the following purification procedures. Thus, after cationization with PEI600-SPDP, possible remaining free SH groups were completely protected by the reaction with a smaller size of APS-sulfonate at 3 mM for another 20 min. After cationization, the mixture was dispersed into 10% acetic acid at pH 3 with vigorous stirring. After removal of insoluble materials by centrifugation, reversibly cationized p53 proteins were concentrated by lyophilization, dissolved in a small amount of Milli-Q water, extensively dialyzed against 0.5% acetic acid at pH 4 to remove excess reagents, and purified by a reversed-phase HPLC column (YMC-Pack ODS-A, YMC, Kyoto, Japan) under a linear-gradient elution of acetonitrile from 30 to 40% in the presence of 0.1% HCl. After exchange of the solvent to phosphate-buffered saline (PBS, pH 7.4) by dialysis or a PD-10 column (Amersham Bioscience, Buckinghamshire, U.K.), the purified reversibly cationized p53 proteins, AP-SS-p53 and PEI600-SS-p53, were used for biological experiments. The number of cysteine residues modified with PEI600 in PEI600-SS-p53 was determined by MALDI−TOF mass spectrometry with a Perspective Voyager-DE PRO mass spectrometer.

As a control protein, bovine serum albumin (BSA) was also reversibly cationized with PEI600-SPDP to give PEI600-SS-BSA, which is a reduced and denatured BSA derivative reversibly cationized with PEI through SS bonds. That is, BSA was reduced with DTT in 0.525 M Tris-HCl (pH 8.6) containing 8 M urea (*6*) and reversibly cationized with PEI600-SPDP for 30 min. In this case, possible remaining SH groups were irreversibly modified by incubation with iodoacetamide for 15 min, and the exchange of the solvent to PBS was carried out by a PD10 column.

Folding Ability of Reversibly Cationized p53 Protein In Vitro. To test the spontaneous folding ability of denatured and reversibly cationized p53 proteins under cytosolic redox conditions, rapid dilution of PEI600-SS-p53 into PBS (pH 7.4) containing 2.5 mM reduced glutathione and 0.05 mM oxidized glutathione at a final protein concentration of 20 μg/mL at 20 °C was carried out. Aliquots were withdrawn at appropriate intervals and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)

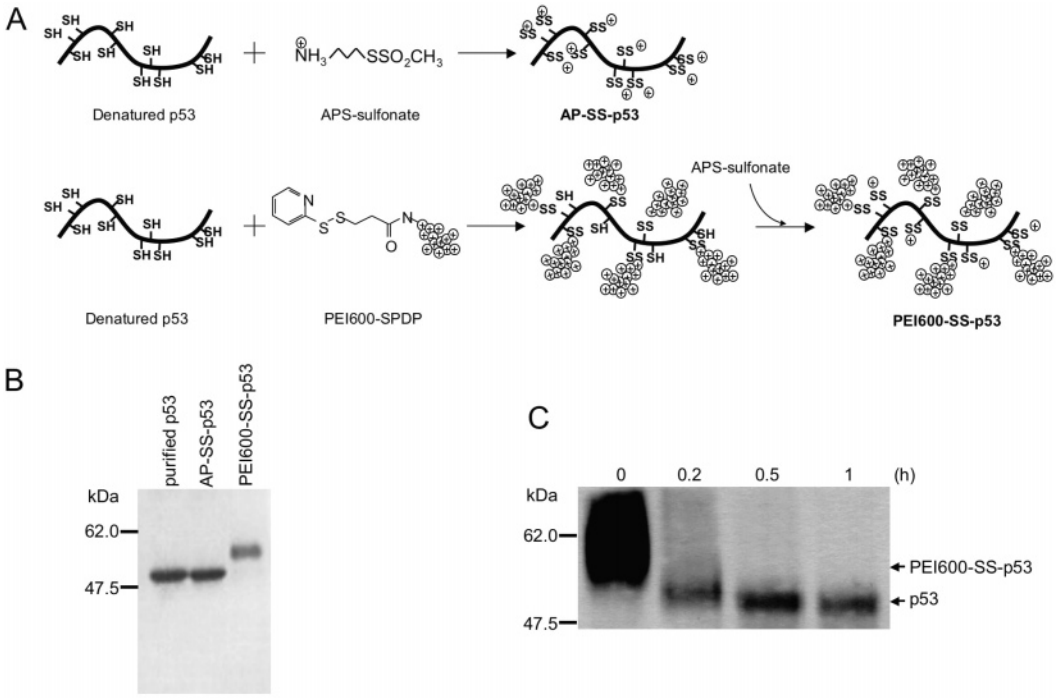

FIGURE 1: Preparation and characterization of denatured and reversibly cationized p53 proteins. (A) Reduced and denatured p53 obtained from inclusion bodies was reversibly cationized in mixed disulfide bonds with APS-sulfonate to give AP-SS-p53 or with PEI600-SPDP followed by APS-sulfonate to give PEI600-SS-p53. (B) SDS−PAGE analysis of purified recombinant p53 and its derivatives (AP-SS-p53 and PEI600-SS-p53) on 10% polyacrylamide gel under nonreducing conditions. The gel was stained with Coomassie Briliant Blue R250. (C) Time-dependent reduction of PEI600-SS-p53 in vitro under cytosolic redox conditions. PEI600-SS-p53 was incubated in a mixture of 2.5 mM reduced glutathione and 0.05 mM oxidized glutathione at pH 7.4 and 20 °C for indicated periods and subjected to SDS−PAGE followed by Western blot analysis using mouse anti-p53 antibody Bp53-12 as a primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG antibody as a secondary antibody. Details are given in the text.

under nonreducing conditions. The gel was blotted onto a nitrocelulose membrane for Western blot analysis using mouse anti-p53 antibody Bp53-12 as a primary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Cell Signaling) as a secondary antibody, and an enhanced chemiluminescence kit (Amersham Biosciences).

Cell Culture and Protein Transduction. p53-null human osteosarcoma Saos-2 and Saos-2/simian virus 40 large-T antigen (SVLT) cells and normal human fibroblast OUMS-24 cells (*12*) were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Sigma). For establishment of Saos-2/SVLT cells, a plasmid encoding SVLT and a G418 resistance marker was introduced into Saos-2 cells using LipofectAMIN 2000 (Invitrogen, CA), and a stably transfected clone was isolated. All cells were cultured in a humidified 5% CO_2 incubator at 37 °C. For protein transduction experiments, about 80% confluent cells were washed once with serum-free medium and then a cationized protein was added under serum-free conditions. After incubation for 1 h at 37 °C, FBS was added at a final concentration of 10% and cells were further incubated for appropriate periods.

Immunoprecipitation and Western Blot Analysis. Cell lysates were prepared using lysis buffer containing 40 mM Tris-HCl at pH 7.4, 1% TritonX-100, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA), 1 mM DTT, and 10% glycerol and were clarified by centrifugation. For immunoprecipitation, cell lysates were precleared with protein G-Sepharose beads (Amersham Biosciences) and then incubated with anti-native p53 antibody Ab-5 for 1 h at 4 °C on a rotating rocker, followed by an additional incubation with protein G-Sepharose beads for 1 h. The immune complexes as well as cell extracts were subjected to SDS−PAGE and transferred onto nitrocellulose membranes. For Western blot analysis, after blocking with 10% skim milk powder and 6% glycine dissolved in PBS, membranes were incubated with anti-p53 antibody Bp53-12, anti-p21/waf1 antibody 6B6, anti-β-actin antibody, or anti-tubulin antibody as primary antibodies. Each specific antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibody and an enhanced chemiluminescence kit as described above.

Cross-Linking Analysis. For analysis of the quaternary structure of exogenously added p53, cells were treated with 1 mM glutaraldehyde for 10 min at 25 °C to preserve the molecular interaction by cross-linking. The reaction was terminated by the addition of 0.1 M glycine. Cell lysates prepared were analyzed by Western blot analysis using anti-p53 antibody Bp53-12. The normal human fibroblast OUMS-24 cells were incubated under confluent or serum-starved conditions for 24 h or the cells were treated with 10 μM 4-nitroquinoline 1-oxide (4NQO) for 6 h. These cells were used as positive controls. Saos-2 and OUMS-24 cells incubated without additives or Saos-2 cells incubated with PEI600-SS-BSA were used as negative controls.

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 exogenously added p53 or endogenously expressed p21/waf1 was performed by treatment of cells with

mouse anti-p53 antibody Bp53-12 or mouse anti-p21/waf1 antibody 187, respectively, followed by treatment with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-mouse IgG antibody (Sigma). Nuclei were stained with Hoechst 33258 (Dojin Laboratories, Kumamoto, Japan). The cells were observed under a fluorescent microscope (IX71-22FL/PH, Olympus).

Northern Blotting. Total RNA was isolated using the guanidium thiocyanate method (*15*). Total RNA was separated by electrophoresis (15 μg/lane) in a 1% formaldehyde-agarose gel and then transferred to a nylon membrane (Nytran-Plus, Schleicher and Schuell, Keene, NH). The blots were probed with gel-purified [γ-^{32}P]dCTP-labeled cDNAs for p21/waf1 mRNA. Membranes were subsequently stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization.

Electrophoresis Mobility Shift Assay. An electrophoresis mobility shift assay was performed as described by Nakano et al. (*16*) using crude nuclear extracts from cells treated with 100 nM PEI600-SS-p53, 100 nM PEI600-SS-BSA, or 10 μM 4NQO for 6 h. We used a double-strand oligonucleotide for the p53-binding element as a probe (Santa Cruz Biotechnology, Inc.). The consensus sequence is 5′-TACA-GAACATGTCTAAGCATGCTGGGG-3′. The [γ-^{32}P]dATP-labeled probe was mixed with crude nuclear extracts of Saos-2 or OUMS-24 cells, incubated for 1 h at 4 °C, and electrophoresed in a 5% polyacrylamide gel under nondenaturing conditions.

Apoptosis Analysis with DNA-Ladder Formation. The cells at various stages were harvested and incubated with lysis buffer containing 10 mM Tris-HCl at pH 7.4, 5 mM EDTA, and 1% TritonX-100 for 20 min on ice. The supernatant containing DNA fragments was incubated with 10 μg/mL RNase A for 1 h at 37 °C. After further incubation with 20 μg/mL proteinase K for 1 h at 37 °C, samples were washed with phenol−chloroform and DNA was precipitated with ethanol. Recovered DNA was electrophoresed on 2% agarose gel and stained with ethidium bromide.

RESULTS

Preparation of Denatured and Reversibly Cationized p53. As summarized in Figure 1A, reversibly cationized p53 proteins were prepared from inclusion bodies dissolved in 6 M guanidine-HCl by alkylsulfidation of sulfhydryl groups with APS-sulfonate and PEI600-SPDP, respectively. Because the modification of p53 with PEI600-SPDP was found to be incomplete (approximately 6 of 10 cysteine residues) probably because of steric hindrance, the remaining 4 free sulfhydryl groups were completely protected with a smaller size of APS-sulfonate. Formation of mixed disulfides with positively charged side-chain groups, especially with PEI, in the polypeptide chain resulted in slower migration on SDS−PAGE under nonreducing conditions (Figure 1B). Both cationized p53 proteins (AP-SS-p53 and PEI600-SS-p53) showed good solubility in water, especially at acidic pH values, because of an increase in the net positive charge by protonation of carboxyl groups. We have confirmed that reversibly cationized p53 proteins remained soluble in 0.5% acetic acid for more than 6 months at 4 °C.

Upon rapid dilution in vitro, reduced p53 purified from inclusion bodies has been reported to have the ability to fold into the biologically active conformation (*17*). Thus, for reversibly cationized p53 proteins to fold in vivo, it is important that they are able to revert to unprotected reduced p53 under cytosol-mimic redox conditions (50:1 reduced glutathione/oxidized glutathione) (*18*). As mentioned above (Figure 1B), PEI600-SS-p53 migrates slower than p53 on SDS−PAGE, and hence, reduction of PEI600-SS-p53 can be monitored. Upon dilution of PEI600-SS-p53 into the redox buffer, PBS (pH 7.4) containing 2.5 mM reduced glutathione and 0.05 mM oxidized glutathione, at a final protein concentration of 20 μg/mL at 20 °C, rapid removal of protecting groups by reduction was observed, and this reduction seemed to be completed in about 1 h to give unprotected p53 (Figure 1C). No precipitation was observed during the process. These results suggest that the reversibly cationized p53 proteins can be reduced to free p53 if they internalized into the cytosol and therefore might fold into the native p53.

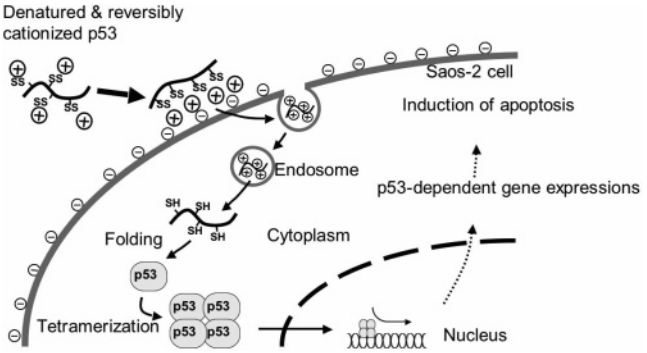

FIGURE 2: Schematic diagram of the "in-cell folding" method for the p53 tumor-suppressor protein. The essential steps for externally added denatured and reversibly cationized p53 to express the native protein functions in Saos-2 cells (electrostatic cell-surface adsorption and/or endosome accumulation, reduction of disulfide bonds because of the release to cytosol, folding and tetramer formation, localization into the nucleus, induction of p53 target genes, and induction of apoptosis of cells) are depicted.

Intracellular Protein Delivery of Reversibly Cationized p53. To investigate whether reversibly cationized p53 proteins could internalize into cells and simultaneously fold into the biologically active structure, we tested essential steps of known p53-dependent cellular responses, as depicted in Figure 2. We employed Saos-2 cells for this assay, because of the lack of endogenous p53 (*19*).

As visualized by immunofluorescence staining, Saos-2 cells showed marked cellular uptake of p53 when exposed to either AP-SS-p53 or PEI600-SS-p53 for 6 h (Figure 3A). Although these fluorescence patterns observed using a fluorescence microscope did not enable p53 internalized in cytoplasmic compartments to be distinguished from the cell-surface associated one, observation of PEI600-SS-p53-treated cells using a confocal laser-scanning microscope indicated that fluorescence was mostly present inside cells (data not shown). The more cationic PEI600-SS-p53 seemed more effective in cellular uptake than did AP-SS-p53 (Figure 3A).

We have previously demonstrated that PEI-cationized proteins enter living cells and have shown that the major mechanism of the protein internalization is endocytotic uptake and subsequent release into the cytosol from endosomes (*4*, *5*). If the reversibly cationized p53 proteins internalize via an endocytotic pathway, the entrapped proteins

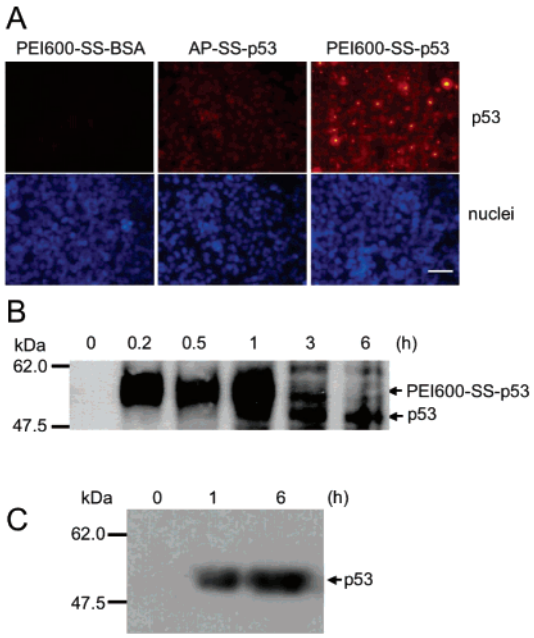

FIGURE 3: Cellular uptake, reduction, and folding of exogenously added reversibly cationized p53 proteins to Saos-2 cells. (A) Saos-2 cells were incubated with 100 nM PEI600-SS-BSA, AP-SS-p53, or PEI600-SS-p53 for 6 h, fixed, immunostained with mouse anti-p53 antibody Bp53-12 and TRITC-conjugated anti-mouse IgG antibody, and observed under a fluorescent microscope (upper panels). Nuclei stained with Hoechst 33258 are also indicated (lower panels). The scale bar is equivalent to 50 μm. (B) Time course of the reduction of PEI600-SS-p53 in cells. Saos-2 cells were incubated with 100 nM PEI600-SS-p53 for indicated periods, and the reduction of PEI600-SS-p53 was analyzed by Western blot analysis with anti-p53 antibody Bp53-12 after SDS−PAGE of cell lysates under nonreducing conditions. (C) Detection of intracellularly folded p53. After incubation of Saos-2 cells with 100 nM PEI600-SS-p53 for indicated periods, folded p53 was collected from cell lysates by immunoprecipitation using anti-native p53 antibody Ab-5 and protein G-Sepharose beads. The precipitates were then electrophoresed by SDS−PAGE under reducing conditions, blotted to nitrocellulose membranes, and analyzed by Western blot analysis with anti-p53 antibody Bp53-12.

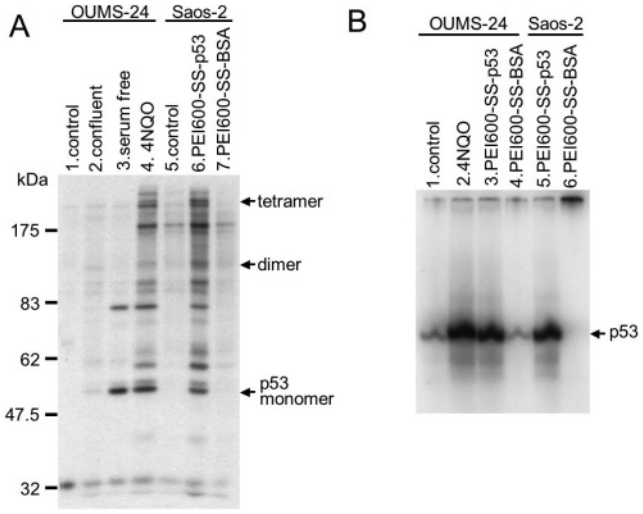

FIGURE 4: Analysis of the quaternary structure and DNA binding of folded p53. (A) Oligomeric states of internalized p53 in cells determined by cross-linking analysis. Normal human fibroblast OUMS-24 cells were incubated under nonconfluent conditions for 6 h (negative control, lane 1), under confluent conditions for 24 h (positive control, lane 2), or under serum-starved conditions for 24 h (positive control, lane 3) or were treated with 10 μM 4NQO for 6 h (positive control, lane 4). p53-null Saos-2 cells were incubated without additives (negative control, lane 5), with 100 nM PEI600-SS-p53 (lane 6), or with PEI600-SS-BSA (negative control, lane 7) for 6 h. The cells were treated with 1 mM glutaraldehyde and then lysed, and cell lysates were analyzed by Western blot analysis using anti-p53 antibody Bp53-12. Arrows indicate positions of the tetramer, dimer, and monomer of p53, which were separately determined by using p53 cross-linked with glutaraldehyde in vitro (data not shown). (B) Electrophoresis mobility shift assay of the p53-binding element with internalized p53 in cells. Crude nuclear extracts from OUMS-24 cells incubated without additives (lane 1), with 10 μM 4NQO (lane 2), with 100 nM PEI600-SS-p53 (lane 3), or with 100 nM PEI600-SS-BSA (lane 4) for 6 h or those from Saos-2 cells incubated with 100 nM PEI600-SS-p53 (lane 5) or 100 nM PEI600-SS-BSA (lane 6) for 6 h were incubated with [γ-^{32}P]dATP-labeled double-strand oligonucleotide for the p53-binding element for 1 h at 4 °C and then electrophoresed in a 5% polyacrylamide gel under nondenaturing conditions. The [γ-^{32}P]dATP-labeled p53-binding element in the gel was detected autoradiographically. Arrow indicates the position of p53.

in the endosome would remain inactive because disulfide bonds are scarcely reduced under endosomal oxidative conditions, but they might fold to the active conformation in the cytosol because disulfide bonds are readily reduced under cytosolic reductive conditions (Figure 1C). Thus, reduction of mixed disulfide bonds in reversibly cationized p53 proteins should be good proof of their cytosolic release. Western blot analysis of SDS−PAGE for PEI600-SS-p53-treated Saos-2 cells indicated that the band corresponding to PEI600-SS-p53 because of cell-surface adsorption and/or endosome accumulation was the main band for the first 1 h of incubation but that the band corresponding to reduced p53 because of cytosolic release became the main band after 3 and 6 h of incubation (Figure 3B).

To confirm time-dependent folding of reversibly cationized p53 proteins in Saos-2 cells, folded p53 in the cell lysate was immunoprecipitated with a specific antibody recognizing the native conformation (anti-native p53 antibody Ab-5) after incubation of cells with 100 nM PEI600-SS-p53 for 1 and 6 h, and the precipitates were analyzed by Western blotting analysis using anti-p53 antibody Bp53-12 (Figure 3C). Time-dependent formation of p53 assuming native conformation was clearly observed, being well-correlated with the reduction of PEI600-SS-p53 in the cytosol (Figure 3B). All of these observations suggest that exogenously added reversibly cationized p53 proteins are rapidly adsorbed on the cell surface by the electrostatic interaction, endocytozed by cells up to 1 h at 37 °C, and more slowly released into the cytosol, where reduction of mixed disulfide bonds followed by folding to the biologically active form takes place.

Quaternary Structure and Specific DNA Binding of Intracellularly Folded p53. The transcriptional activity of p53 has been reported to be associated with a tetrameric form that binds DNA in a sequence-specific fashion to activate the transcription of target genes (*20*−*23*). To confirm the formation of tetrameric p53 when delivered into cells as described above, we performed cross-linking experiments with glutaraldehyde as a cross-linking agent to fix the protein quaternary structure. As shown in Figure 4A, the electrophoretic pattern obtained from PEI600-SS-p53-treated Saos-2 cells indicated the presence of a covalently cross-linked p53 dimer and tetramer including possibly their degraded products (lane 6). No such pattern was obtained from Saos-2 cells treated without cationized protein (one negative control, lane

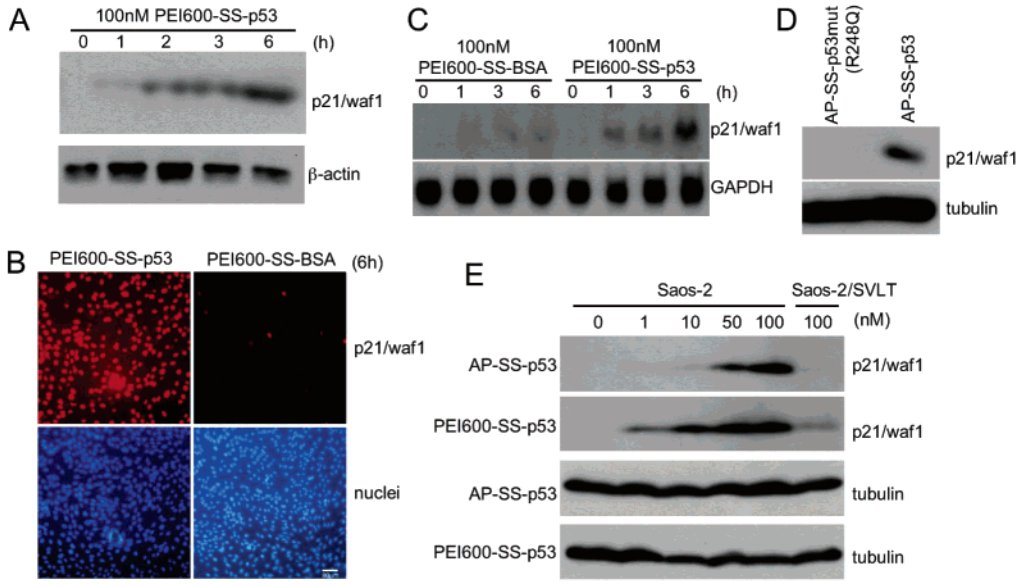

FIGURE 5: Induction of the p53-dependent gene expression of p21/waf1. Saos-2 cells were incubated with 100 nM PEI600-SS-p53 or PEI600-SS-BSA for indicated periods, and induction of p21/waf1 was examined at the protein level (A and B) or mRNA level (C). (A) Western blot analysis of the time-dependent expression of the p21/waf1 protein in Saos-2 cells by treatment with 100 nM PEI600-SS-p53. Expression of p21/waf1 (upper panel) was examined by SDS−PAGE of cell lysates and Western blot analysis with anti-p21/waf1 antibody 6B6. The expression level of β-actin (lower panel) was examined by reprobing using anti-β-actin antibody for normalization. (B) Saos-2 cells were incubated with 100 nM PEI600-SS-p53 (left panels) or PEI600-SS-BSA (right panels) for 6 h, fixed, immunostained with mouse anti-p21/waf1 antibody 187 and TRITC-conjugated anti-mouse IgG antibody, and then observed under a fluorescent microscope (upper panels). Nuclei stained with Hoechst 33258 are also indicated (lower panels). The bar indicates 80 μm. (C) Northern blot analysis for examination of the time-dependent expression of p21/waf1 mRNA in PEI600-SS-p53-treated Saos-2 cells. Total RNAs isolated from cells treated with 100 nM PEI600-SS-BSA or PEI600-SS-p53 for indicated periods were separated by electrophoresis and transferred to nylon membranes. The blots were probed with [γ-^{32}P]dCTP-labeled cDNAs for p21/waf1 (upper panel). Membranes were subsequently stripped and reprobed for GAPDH for normalization (lower panel). (D) Transactivation activity of the p53-dependent p21/waf1 protein expression. Saos-2 cells were incubated with 100 nM AP-SS-p53 (upper panel, right) or transactivation-deficient AP-SS-p53 mutant [AP-SS-p53mut (R248Q)] (upper panel, left) for 6 h and analyzed by Western blot analysis using anti-p21/waf1 antibody 6B6. Tubulin was employed for normalization (lower panel). (E) Dose dependence of p21/waf1 protein expression in reversibly cationized p53-treated Saos-2 cells and lack of p53 function in reversibly cationized p53-treated Saos-2/SVLT cells. Saos-2 cells and Saos-2/SVLT cells that express SVLT suppressing the p53 function were incubated with AP-SS-p53 or PEI600-SS-p53 at indicated concentrations for 6 h. After SDS−PAGE of cell lysates, expression levels of p21/waf1 were analyzed by Western blot analysis using anti-p21/waf1 antibody 6B6 (top panel for AP-SS-p53 and second-to-top panel for PEI600-SS-p53). Tubulin was employed for normalization (bottom and second-to-bottom panels).

5) or from Saos-2 cells treated with cationized BSA (another negative control, lane 7). However, the same pattern was obtained from normal OUMS-24 cells that the endogenous p53 level was upregulated by 4NQO (a positive control, lane 4) (*12*, *24*). These results suggest that externally added reversibly cationized p53 folds intracellularly to a tetrameric form. Interestingly, the pattern obtained from confluent OUMS-24 cells (one of the positive controls, lane 2) seemed similar to the pattern from PEI600-SS-p53-treated cells, although the level of tetrameric p53 was very low, while the pattern obtained from serum-starved OUMS-24 cells (another positive control, lane 3) seemed different from those of other positive controls in the distribution of p53 oligomers and degraded products. The growth-arrested state in serum-starved cells might not be the same as that in confluent cells.

Next, we investigated the specific binding ability of intracellularly folded p53 to a DNA fragment of the p53-binding element. As shown in Figure 4B, the nuclear extract obtained from Saos-2 cells treated with PEI600-SS-p53 for 6 h contained p53 showing specific binding activity against the labeled DNA fragment (lane 5). Similar results were also obtained by using nuclear extracts from 4NQO-treated OUMS-24 cells (positive control, lane 2) and from PEI600-SS-p53-treated OUMS-24 cells (lane 3). The nuclear extract from OUMS-24 cells incubated without cationized protein (lane 1) or with cationized BSA (lane 4) showed a background level of active p53 in the cells. The nuclear extract from cationized BSA-treated Saos-2 cells did not give the band corresponding to p53 (lane 6). These results imply that the intracellularly folded p53 was translocated to the nucleolus as a biologically active tetramer when cells were incubated with PEI600-SS-p53 for 6 h.

Induction of Intracellularly Delivered p53-Dependent Gene Expression. If exogenously added reversibly cationized p53 proteins express their transcriptional activity, one of the known p53 target gene products, p21/waf1, should be upregulated in Saos-2 cells (*20*, *25*). Thus, we examined the effect of treatment of Saos-2 cells with AP-SS-p53 or PEI600-SS-p53 on the expression of p21/waf1 and found that was the case (Figure 5). Time-dependent expression of p21/waf1 was examined by adding 100 nM PEI600-SS-p53 to the culture medium of Saos-2 cells. The cells were harvested several times for Western blot analysis up to 6 h after the addition of PEI600-SS-p53. The protein level of p21/waf1 was gradually elevated by this treatment (Figure 5A). Immunofluorescence experiments revealed that the expression of p21/waf1 was induced in approximately 60% of cells by this treatment for 6 h but not by treatment with PEI600-SS-BSA (Figure 5B). The same was true when the induction of p21/waf1 was monitored by the mRNA level (Figure 5C). Another reversibly cationized p53 protein, AP-SS-p53, also induced p21/waf1 expression in Saos-2 cells,

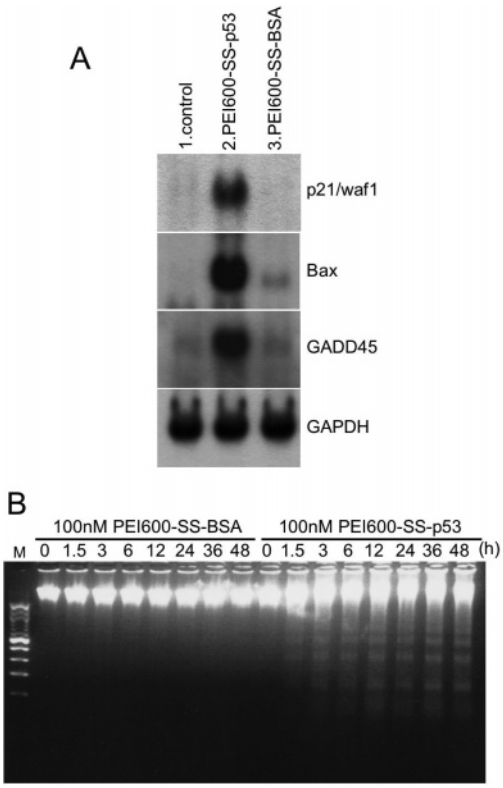

FIGURE 6: Biological effects of introduced p53 on Saos-2 cells. (A) Saos-2 cells were treated without additives (control, lane 1), with 100 nM PEI600-SS-p53 (lane 2), or with PEI600-SS-BSA (lane 3) for 6 h, and transcriptional activation of p53-dependent apoptotic genes (p21/waf1, Bax, and GADD45) was examined by Northern blot analysis. GAPDH was employed for normalization. (B) DNA fragmentation in Saos-2 cells treated with PEI600-SS-p53. After incubation of Saos-2 cells with 100 nM PEI600-SS-p53 or PEI600-SS-BSA for indicated periods, the respective DNAs were extracted and analyzed by electrophoresis on a 2% agarose gel. The gel was stained with ethidium bromide. M indicates molecular markers (the left most lane).

but p21/waf1 expression was not induced when cells were treated with reversibly cationized transactivation-deficient AP-SS-p53 mutant (R248Q) (*26*) (Figure 5D). The latter result is consistent with the failure of AP-SS-p53 or PEI600-SS-p53 to induce p21/waf1 in Saos-2/SVLT cells that express SVLT suppressing p53 function by specific binding (*27*) (Figure 5E). We further compared AP-SS-p53 and PEI600-SS-p53 for dose dependency to the induction of p21/waf1. The more cationic PEI600-SS-p53 showed more efficient activity than did AP-SS-p53 as seen in the treatment with 1 nM for 6 h (Figure 5E). These results demonstrated that exogenous p53 delivered by this method is a highly potent inducer of the target gene expression.

Induction of Apoptosis by Intracellularly Delivered p53-Dependent Gene Expression. Because it has been reported that induction of p53 expression in Saos-2 cells at a low level induces growth arrest but at a high level induces apoptosis (*28*), we analyzed apoptotic gene expression in PEI600-SS-p53-treated Saos-2 cells. Treatment with 100 nM PEI600-SS-p53 but not with 100 nM PEI600-SS-BSA for 6 h caused transcriptional activation of p53-dependent apoptotic genes (p21/waf1, Bax, and GADD45) in Saos-2 cells (Figure 6A). These results were supported by apoptotic DNA-ladder formation in PEI600-SS-p53-treated cells compared with that in PEI600-SS-BSA-treated cells (Figure 6B).

DISCUSSION

The living cell membrane poses a substantial hurdle for exogenous protein to express its function inside cells. To overcome this limitation, a variety of new methodologies to deliver functional proteins into cells are being studied actively. The new methodologies include "protein cationization methods". Methods using 8−35 amino-acid-long peptides called "protein transduction domains" (PTDs) are the most popular (*29*−*35*). PTDs derived from human immunodeficiency virus-transactivator of transcription (HIV-TAT) (*30*, *32*), herpes simplex virus-structural protein VP22 (HSV-VP22) (*31*), and antennapedia (*29*) or synthetic PTDs (*33*−*35*) are characterized by a high content of positively charged arginine and lysine residues, which are potentially important for association with the negatively charged cell-surface membrane by electrostatic interaction followed by internalization into cells by endocytosis (*36*, *37*) or macropinocytosis (*38*). Chemical cationization methods developed by us (*3*−*5*) are also included in this category. A cationic lipid-based carrier system (*39*), virus envelope vector (*40*), and PEI-cationized carrier systems (*5*) have also been proposed. Although these current protein transduction techniques seem promising for laboratory investigations and therapeutic applications, proteins to be delivered into cells require sufficient solubility. Because denatured proteins are hardly soluble in physiological aqueous solutions, detergents or denaturants toxic for living cells are generally used to solubilize them. In the present study, we showed that the denatured form of the reversibly cationized p53 protein was not only soluble in physiological solutions but also active for transduction into living cells to express its functions inside cells because of simultaneous folding. We call this protein transduction method the "in-cell folding" technique. This technique would be of great value especially when proteins to be delivered into cells are not easily available as biologically active conformations in vitro.

Because mutations in p53 are among the most common genetic events in the development of human cancer (*41*), many studies have been carried out to evaluate viral vector-mediated p53 gene delivery for gene therapy (*42*). Approaches for protein transduction therapy using a PTD peptide fused to folded p53 have also been carried out and have been shown to be useful to inhibit the proliferation of cancer cells (*43*−*45*). Although a protein transduction method allows the protein to function in cells transiently, PEI600-SS-p53 could induce p53-target genes only at 1 nM (Figure 5E), suggesting that the method would be effective at limited doses to inhibit cancer cell growth in therapeutic applications.

In this study, we used APS-sulfonate and PEI600-SPDP to cationize denatured p53 (Figure 1A). Both reagents possess moieties with common characteristics, a SH-selective and rapid modifying moiety (methanethiosulfonyl or dithiopyridyl group) and a cationic side-chain moiety (amine or polyamine group). Thus, in the reaction with these reagents, positive charges were introduced into 10 cysteine residues (that is, 10 free SH groups) of p53 through the formation of mixed disulfide bonds to give AP-SS-p53 or PEI600-SS-p53. As shown in Figure 1C, AP-SS-p53 and PEI600-SS-p53 were equivalent to reduced p53 in the SS−SH interchange reaction under cytosolic redox conditions. In other words, denatured

AP-SS-p53, PEI600-SS-p53, and reduced p53 have equivalent folding abilities in the cytosol.

If charged residues at neutral pH are assumed to be only Asp(−1), Glu(−1), Lys(+1), and Arg(+1) for simplicity, the net charge of wild-type p53 is calculated to be −4. APS-sulfonate and PEI600-SPDP can introduce positive charges of +1 and +13.6, respectively, into every cysteine residue. As mentioned above, PEI600-SS-p53 contained approximately six and four cysteine residues modified with PEI600-SPDP and APS-sulfonate, respectively. Thus, net charges of AP-SS-p53 and PEI600-SS-p53 were calculated to be +6 and +81.6, respectively. We previously showed that the net positive charge of proteins correlated well with their efficiency in protein transduction (*2*, *3*, *46*). Consistent with this previous observation, PEI600-SS-p53 seemed to be more effective than AP-SS-p53 (Figures 3A and 5E).

Although various recombinant protein production systems or in vitro translation systems are now available, there is no guarantee that a large amount of biologically active products will be yielded. However, denatured proteins can be easily obtained as inclusion bodies in good yield if an *E. coli* expression system is used. The results presented here demonstrated the possibility of the "in-cell folding" technique; that is, reversibly cationized unfolded proteins could internalize into living cells and simultaneously fold to biologically active conformations. Because many newly synthesized proteins in the cytosol require involvement of complex cellular machinery such as chaperones and processing enzymes as well as input of metabolic energy to reach their native states (*47*) and because such machinery is not easily utilized in vitro, the strategy of "in-cell folding" may be reasonable in some cases. Thus, the "in-cell folding" technique may greatly enhance the utility of a protein expression system, yielding unfolded proteins such as hardly soluble inclusion bodies when protein transduction into living cells is attempted.

REFERENCES

1. Kumagai, A. K., Eisenberg, J. B., and Pardridge, W. M. (1987) Absorptive-mediated endocytosis of cationized albumin and a β-endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport, *J. Biol. Chem. 262*, 15214−15219.
2. Futami, J., Maeda, T., Kitazoe, M., Nukui, E., Tada, H., Seno, M., Kosaka, M., and Yamada, H. (2001) Preparation of potent cytotoxic ribonucleases by cationization: Enhanced cellular uptake and decreased interaction with ribonuclease inhibitor by chemical modification of carboxyl groups, *Biochemistry 40*, 7518−7524.
3. Futami, J., Nukui, E., Maeda, T., Kosaka, M., Tada, H., Seno, M., and Yamada, H. (2002) Optimum modification for the highest cytotoxicity of cationized ribonuclease, *J. Biochem. 132*, 223−228.
4. Futami, J., Kitazoe, M., Maeda, T., Nukui, E., Sakaguchi, M., Kosaka, J., Miyazaki, M., Kosaka, M., Tada, H., Seno, M., Sasaki, J., Huh, N. H., Namba, M., and Yamada, H. (2005) Intracellular delivery of proteins into mammalian living cells by polyethylenimine-cationization, *J. Bioeng. Biosci. 99*, 95−103.
5. Kitazoe, M., Murata, H., Futami, J., Maeda, T., Sakaguchi, M., Miyazaki, M., Kosaka, M., Tada, H., Seno, M., Huh, N. H., Namba, M., Nishikawa, M., Maeda, Y., and Yamada, H. (2005) Protein transduction assisted by polyethylenimine-cationized carrier proteins, *J. Biochem. 137*, 693−701.
6. Yamada, H., Seno, M., Kobayashi, A., Moriyama, T., Kosaka, M., Ito, Y., and Imoto, T. (1994) An *S*-alkylating reagent with positive charges as an efficient solubilizer of denatured disulfide-containing proteins, *J. Biochem. 116*, 852−857.
7. Inoue, M., Akimaru, J., Nishikawa, T., Seki, N., and Yamada, H. (1998) A new derivatizing agent, trimethylammoniopropyl methanethiosulphonate, is efficient for preparation of recombinant brain-derived neurotrophic factor from inclusion bodies, *Biotechnol. Appl. Biochem. 28*, 207−213.
8. Seno, M., DeSantis, M., Kannan, S., Bianco, C., Tada, H., Kim, N., Kosaka, M., Gullick, W. J., Yamada, H., and Salomon, D. S. (1998) Purification and characterization of a recombinant human cripto-1 protein, *Growth Factors 15*, 215−229.
9. Mallorqui-Fernandez, G., Pous, J., Peracaula, R., Aymami, J., Maeda, T., Tada, H., Yamada, H., Seno, M., de Llorens, R., Gomis-Ruth, F. X., and Coll, M. (2000) Three-dimensional crystal structure of human eosinophil cationic protein (RNase 3) at 1.75 Å resolution, *J. Mol. Biol. 300*, 1297−1307.
10. Miura, K., Doura, H., Aizawa, T., Tada, H., Seno, M., Yamada, H., and Kawano, K. (2002) Solution structure of betacellulin, a new member of EGF-family ligands, *Biochem. Biophys. Res. Commun. 294*, 1040−1046.
11. Newton, D. L., Futami, J., Ruby, D., and Rybak, S. M. (2003) Construction and characterization of RNase-based targeted therapeutics, *Methods Mol. Biol. 207*, 283−304.
12. Bai, L., Mihara, K., Kondo, Y., Honma, M., and Namba, M. (1993) Immortalization of normal human fibroblasts by treatment with 4-nitroquinoline 1-oxide, *Int. J. Cancer 53*, 451−456.
13. Futami, J., Tsushima, Y., Tada, H., Seno, M., and Yamada, H. (2000) Convenient and efficient in vitro folding of disulfide-containing globular protein from crude bacterial inclusion bodies, *J. Biochem. 127*, 435−441.
14. Futami, J., Tada, H., Seno, M., Ishikami, S., and Yamada, H. (2000) Stabilization of human RNase 1 by introduction of a disulfide bond between residues 4 and 118, *J. Biochem. 128*, 245−250.
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, *Biochemistry 18*, 5294−5299.
16. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H., and Sakai, T. (1997) Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line, *J. Biol. Chem. 272*, 22199−22206.
17. Bell, S., Hansen, S., and Buchner, J. (2002) Refolding and structural characterization of the human p53 tumor suppressor protein, *Biophys. Chem. 96*, 243−257.
18. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum, *Science 257*, 1496−1502.
19. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B., and Friend, S. H. (1990) p53 functions as a cell cycle control protein in osteosarcomas, *Mol. Cell. Biol. 10*, 5772−5781.
20. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression, *Cell 75*, 817−825.
21. Halazonetis, T. D., and Kandil, A. N. (1993) Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants, *EMBO J. 12*, 5057−5064.
22. Friedman, P. N., Chen, X., Bargonetti, J., and Prives, C. (1993) The p53 protein is an unusually shaped tetramer that binds directly to DNA, *Proc. Natl. Acad. Sci. U.S.A. 90*, 3319−3323.
23. Stenger, J. E., Tegtmeyer, P., Mayr, G. A., Reed, M., Wang, Y., Wang, P., Hough, P. V., and Mastrangelo, I. A. (1994) p53 oligomerization and DNA looping are linked with transcriptional activation, *EMBO J. 13*, 6011−6020.
24. Mirzayans, R., Bashir, S., Murray, D., and Paterson, M. C. (1999) Inverse correlation between p53 protein levels and DNA repair efficiency in human fibroblast strains treated with 4-nitroquinoline 1-oxide: Evidence that lesions other than DNA strand breaks trigger the p53 response, *Carcinogenesis 20*, 941−946.
25. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage, *Science 282*, 1497−1501.
26. Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) Crystal structure of a p53 tumor suppressor−DNA complex: Understanding tumorigenic mutations, *Science 265*, 346−355.
27. Lane, D. P., and Crawford, L. V. (1979) T antigen is bound to a host protein in SV40-transformed cells, *Nature 278*, 261−263.

28. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. (1996) p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells, *Genes Dev. 10*, 2438−2451.
29. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) The third helix of the *Antennapedia* homeodomain translocates through biological membranes, *J. Biol. Chem. 269*, 10444−10450.
30. Vives, E., Brodin, P., and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, *J. Biol. Chem. 272*, 16010−16017.
31. Elliott, G., and O'Hare, P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein, *Cell 88*, 223−233.
32. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) In vivo protein transduction: Delivery of a biologically active protein into the mouse, *Science 285*, 1569−1572.
33. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13003−13008.
34. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery, *J. Biol. Chem. 276*, 5836−5840.
35. Matsushita, M., Tomizawa, K., Moriwaki, A., Li, S. T., Terada, H., and Matsui, H. (2001) A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation, *J. Neurosci. 21*, 6000−6007.
36. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, *J. Biol. Chem. 278*, 585−590.
37. Lundberg, M., Wikstrom, S., and Johansson, M. (2003) Cell surface adherence and endocytosis of protein transduction domains, *Mol. Ther. 8*, 143−150.
38. Wadia, J. S., Stan, R. V, and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, *Nat. Med. 10*, 310−315.
39. Zelphati, O., Wang, Y., Kitada, S., Reed, J. C., Felgner, P. L., and Corbeil, J. (2001) Intracellular delivery of proteins with a new lipid-mediated delivery system, *J. Biol. Chem. 276*, 35103−35110.
40. Yuki, S., Kondo, Y., Kato, F., Kato, M., and Matsuo, N. (2004) Noncytotoxic ribonuclease, RNase T1, induces tumor cell death via hemagglutinating virus of Japan envelope vector, *Eur. J. Biochem. 271*, 3567−3572.
41. Vogelstein, B., and Kinzler, K. W. (1992) p53 function and dysfunction, *Cell 70*, 523−526.
42. Verma, I. M., and Somia, N. (1997) Gene therapy—Promises, problems and prospects, *Nature 389*, 239−242.
43. Takenobu, T., Tomizawa, K., Matsushita, M., Li, S. T., Moriwaki, A., Lu, Y. F., and Matsui, H. (2002) Development of p53 protein transduction therapy using membrane-permeable peptides and the application to oral cancer cells, *Mol. Cancer Ther. 1*, 1043−1049.
44. Zender, L., Kuhnel, F., Kock, R., Manns, M., and Kubicka, S. (2002) VP22-mediated intercellular transport of p53 in hepatoma cells *in vitro* and *in vivo*, *Cancer Gene Ther. 9*, 489−496.
45. Michiue, H., Tomizawa, K., Wei, F. Y., Matsushita, M., Lu, Y. F., Ichikawa, T., Tamiya, T., Date, I., and Matsui, H. (2005) The NH_2 terminus of influenza virus hemagglutinin-2 subunit peptides enhances the antitumor potency of polyarginine-mediated p53 protein transduction, *J. Biol. Chem. 280*, 8285−8289.
46. Maeda, T., Mahara, K., Kitazoe, M., Futami, J., Takidani, A., Kosaka, M., Tada, H., Seno, M., and Yamada, H. (2002) RNase 3 (ECP) is an extraordinarily stable protein among human pancreatic-type RNases, *J. Biochem. 132*, 737−742.
47. Hartl, F. U., and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: From nascent chain to folded protein, *Science 295*, 1852−1858.

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