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Transient Cell Proliferation with Polyethylenimine-Cationized N-Terminal Domain of Simian Virus 40 Large T-Antigen

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Polyethylenimine (PEI) cationization is a powerful strategy for protein transduction into cells. In this study, we attempted the artificial regulation of cell proliferation by protein transduction of the N-terminal domain (1–132 amino acids) of the simian virus 40 large T-antigen (SVLT-N), which inactivates retinoblastoma family proteins but not p53. To deliver SVLT-N into cells, we employed an indirect cationization method by forming a complex of biotynylated SVLT-N through disulfide bonds (biotin-SS-SVLT-N) and PEI-cationized avidin (PEI600-avidin). Using this complex, SVLT-N was transduced into the nucleus of confluent and quiescent Balb/c 3T3 cells and was found to be complexed with a cellular target protein, pRb. Furthermore, SVLT-N transduction induced cell proliferation in spite of confluent conditions. Because SVLT-N thus transduced into cells gradually degraded and was not detectable after a 4-d incubation, transiently transformed cells were obtained by this method. These results suggest that oncogene protein transduction technology has great potential for *in vitro* regulation of cell proliferation.

[**Key words:** N-terminal domain of simian virus 40 large T-antigen, polyethylenimine cationization, protein transduction, transient cell proliferation, avidin]

Although autologous cell-based therapy is promising for treatment of various disorders, it will remain a theoretical prospect unless the requirement for providing a large number of therapeutic cells can be met. Therefore, technology for controllable expansion of primary cells provides an attractive means of producing a large number of cells for therapeutic purposes. A prototype approach for the induction of proliferation and extension of the life span of primary cultured cells is introduction of a cell cycle inducer or elimination of a negative regulator. One of the most commonly used agents is the simian virus 40 large T-antigen (SVLT), which inactivates the retinoblastoma family (pRb, p107, and p130) and the tumor suppressor p53 (1). Transfer of oncogenes such as SVLT can generate indefinitely proliferative cells, known as cell immortalization. However, *in vivo* injection of immortalized cell lines would expose patients to an unacceptable tumorigenic risk (2). Therefore, a reversible immortalization procedure that involves a retrovirus-mediated transfer of an oncogene that can be subsequently excised by site-specific recombination such as Cre recombinase has been proposed (3).

Recently developed protein transduction technology has preferable safety characteristics for controlling cellular growth and differentiation for cell-based therapy, because transiently transduced proteins will degrade after expression of their function in cells without any alternation of genomic DNA (4–10). A number of efficient protein transduction vehicles, including cationic cell-penetrating peptides (CPPs) and cationic lipid-based reagents, have been proposed. All of these vehicles appear to transduce the target protein through an endocytosis-like pathway after electrostatic adsorption to the negatively charged cellular surface (11, 12). Chemical protein cationization by a synthetic cationic polymer of polyethylenimine (PEI) is also a powerful strategy for protein transduction, because highly cationic proteins rapidly bind to the cellular surface followed by internalization into cells via adsorptive mediated endocytosis (4, 13–15).

In this study, we developed an efficient *in vitro* method for inducing cell proliferation by transient transduction of an oncogenic protein for future cell-based therapy. We employed SVLT as a prototype oncogenic protein and found that the N-terminal domain of 132 amino acids (SVLT-N), which inactivates pRB but not p53, was efficiently delivered into quiescent cells by PEI-cationized carrier proteins and induced cell proliferation. The protein transduction technology of SVLT-N has great potential for *in vitro* regulation of cell proliferation and may pave a new way for *ex vivo* cell expansion needed for cell-based therapy.

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MATERIALS AND METHODS

Materials PEI (Epomin SP series: manufactured by Nippon Shokubai, Osaka) with an average molecular mass of 600 (PEI600) was donated by Nippon Shokubai or purchased from Wako Chemical (Osaka). A plasmid encoding full-length SVLT (pSV3-Neo) was kindly provided by Dr. N. Kobayashi (Okayama University).

Plasmid constructs An expression vector for SVLT-N was constructed by the following amplification and ligation steps. The coding region for the N-terminal domain (1–132 amino acids) of SVLT was amplified from pSV3-Neo by PCR and the fragment obtained was inserted between the *Nde*I and *Bam*HI sites of an over-expression vector, pET14b (Novagen, Madison, WI, USA). The expression vector for an SVLT-N mutant (E107K and E108K, SVLT-mut) was obtained by mutation PCR of the SVLT-N expression vector.

Protein expression and purification Recombinant His-tagged SVLT-N and SVLT-mut were expressed in *Escherichia coli* (*E. coli*) BL21(DE3)plysS (Novagen). Transformed *E. coli* cells were cultured in Terrific Broth (TB) containing 200 μg/ml ampicillin at 37°C. When the optical density of the medium at 600 nm had reached 0.8, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside was added and the cells were cultured for another 3 h. Harvested *E. coli* cells were then lysed by repeating a freeze/thaw/sonication cycle twice. To remove nucleic acids, PEI600 (10% solution that had been adjusted to pH 8 with HCl) was added slowly with stirring on ice until the final concentration was 0.7%. After stirring on ice for 15 min, the resulting precipitates were removed by centrifugation at 10,000×*g* for 15 min. His-tagged proteins were purified using a TALON Superflow metal affinity column (Clontech, Mountain View, CA, USA). Briefly, the supernatant was applied to the column, nonspecifically bound proteins were washed out with PBS containing 0.1% Tween-20 and 5 mM imidazole, and then specifically bound His-tagged proteins were eluted with 50 mM phosphate buffer (pH 7.3) containing 150 mM imidazole and 150 mM NaCl. SVLT-N and SVLT-mut were subsequently purified by ion-exchange HPLC using a RESOURSE-Q column (GE Healthcare, Buckinghamshire, UK) and eluted under a linear gradient of NaCl from 0 to 1.0 M in 50 mM phosphate buffer (pH 7.3) for 80 min at a flow rate of 0.5 ml/min.

Chemical modification Because SVLT-N and SVLT-mut possess two reactive sulfhydryl groups, they were biotinylated through disulfide (SS) bonds with biotin-HPDP (Pierce, Rockford, IL, USA) dissolved in dimethylformamide (DMF) at a molar ratio of biotinylating reagent : protein of 10 : 1 to obtain biotin-SS-SVLT-N and biotin-SS-SVLT-mut, respectively. After incubation for 1 h at room temperature, the biotinylated proteins were fractionated by gel filtration on a PD-10 column (GE Healthcare, 1.5×5 cm) in PBS. Fluorescence-labeling reaction to obtain fluorescein-labeled biotin-SS-SVLT-N was carried out in accordance with the method described previously (16). Coupling reaction of chicken avidin with PEI600 induced with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) was described previously (13). Briefly, a protein was dissolved at a concentration of 1.0 mg/ml in PEI600 solution (60 mg/ml, pH adjusted to 5.0 with HCl). The reaction was initiated by adding solid EDC at 0.1 mg/ml to the protein solution at room temperature to obtain PEI600-avidin. After incubation for 16 h, PEI600-avidin was fractionated by gel filtration on a Sephadex G-25 medium column (GE Healthcare, 1.5×30 cm) in PBS. Protein cationization by PEI was verified by SDS–PAGE.

Cell culture, protein transduction, and DNA transfection Murine fibroblast Balb/c 3T3 cells (Dainippon Pharmaceutical, Tokyo) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 70 μg/ml kanamycin.

Protein transduction assay of quiescent cells was carried out using confluent cell culture arrested in the G0/G1 phase by contact inhibition. When cell growth had reached confluence, the medium was changed to fresh DMEM containing 10% FBS with or without a mixture of 200 nM PEI600-Avidin and 100 nM biotinylated protein. In DNA transfection assay, the medium of confluent cell culture was changed to Opti-MEM (Invitrogen, Carlsbad, CA, USA), and plasmid DNA encoding SVLT-N was transfected with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Six hours after DNA transfection, the medium was changed to DMEM containing 10% FBS.

Western blot analysis Cells were washed twice with ice-cold PBS, and their protein extracts were subjected to Western blot analysis as described previously (15). The primary antibodies used were a mouse anti-SV40 T-antigen antibody (Ab-1; Calbiochem, La Jolla, CA, USA), a mouse anti-Rb (a.a. 332–344) antibody (Becton Dickinson, San Jose, CA, USA), a mouse anti-β-actin antibody (Sigma, St. Louis, MO, USA), a rabbit anti-p27 antibody (c-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit anti-cyclin A antibody (c-19; Santa Cruz Biotechnology). The secondary antibodies used were a horseradish peroxidase-conjugated goat anti-mouse IgG antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology, Beverly, MA, USA). Positive signals were visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer LAS, Boston, MA, USA).

Immunocytochemistry Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 70% ethanol for 1 h at −20°C. Immunofluorescence staining of SVLT-N was performed by treating cells with the anti-SV40 T-antigen antibody (Ab-1; Calbiochem) followed by an Alexa Fluor 488 rabbit anti-mouse IgG antibody (Invitrogen). In bromodeoxyuridine (BrdU) incorporation assay, after the cells had been incubated for 1 h with 10 μM BrdU, they were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and then permeabilized with 100% methanol for 1 h at −20°C. After washing with PBS, the cells were incubated with 2 M HCl for 30 min to denature DNA and then incubated in 1 M Tris–HCl (pH 8) for 1 h to neutralize the acid. For immunodetection of the incorporated BrdU, the fixed cells were incubated with a solution containing an anti-BrdU-fluorescein antibody (Roche, Indianapolis, IN, USA). Nuclei were also stained with Hoechst 33258 (Dojin Laboratories, Kumamoto). The cells were observed under a confocal laser-scanning microscope (model LSM 510; Carl Zeiss, Jena, Germany).

Binding assay of SVLT-N to pRb Approximately 300 μg of cell lysate from culture of confluent Balb/c 3T3 cells was incubated with 10 μg of SVLT-N or SVLT-mut for 1 h at 4°C, and then the mixture was incubated with 30 μl of TALON Superflow metal affinity resin for 1 h at 4°C to precipitate SVLT-N and associated proteins. After centrifugation, the pellets were washed three times with phosphate buffer (pH 7.4) containing 5 mM imidazole, 300 mM NaCl and 3% glycerol, and then adsorbed proteins were dissociated with elution buffer containing 150 mM imidazole. After centrifugation of the eluted fraction, the supernatants were subjected to Western blot analysis.

RESULTS AND DISCUSSION

Intracellular delivery of SVLT-N proteins Initially, we investigated the efficiency of delivery of SVLT-N proteins into Balb/c 3T3 cells *in vitro*. PEI cationization is a useful method for internalizing a protein into living cells via adsorptive-mediated endocytosis. Conjugation of a protein with PEI can be achieved by either direct conjugation of PEI to the molecular surface of the protein (13) or indirect