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Protein transduction of an antioxidant enzyme: subcellular localization of superoxide dismutase fusion protein in cells

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In protein therapy, it is important for exogenous protein to be delivered into the target subcellular localization. To transduce a therapeutic protein into its specific subcellular localization, we synthesized nuclear localization signal (NLS) and membrane translocation sequence signal (MTS) peptides and produced a genetic in-frame SOD fusion protein. The purified SOD fusion proteins were efficiently transduced into mammalian cells with enzymatic activities. Immunofluorescence and Western blot analysis revealed that the SOD fusion proteins successfully transduced into the nucleus and the cytosol in the cells. The viability of cells treated with paraquat was markedly increased by the transduced fusion proteins. Thus, our results suggest that these peptides should be useful for targeting the specific localization of therapeutic proteins in various human diseases. [BMB reports 2008; 41(2): 170-175]

INTRODUCTION

Delivering proteins with therapeutic potential into cells is difficult due to their size and biochemical properties. Thus, it has been problematic to utilize such proteins as therapeutic drugs (1). The therapeutic application of proteins could be achieved by developing delivery vectors that are capable of efficiently delivering proteins into cells.

Many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology (2-12). Several small regions of proteins, called protein transduction domains [PTDs; also known as cell-penetrating peptides (CPPs)], have been developed to allow the delivery of exogenous proteins into living cells. These include carrier peptides derived from the HIV-1 Tat protein, *Drosophila* Antennapedia (Antp) protein, and the herpes simplex virus VP22 protein (13-15). In a previous study, we successfully transduced Tat-SOD directly into various cell lines, including pancreatic islet cells, and found that the transduced Tat-SOD had increased radical scavenging activity (16). Recently, we reported on the protective effects of transduced PEP-1-SOD against neuronal cell death in astrocytes and Parkinson's disease in mouse models induced by paraquat. The protective effect was synergistically increased by co-transduction with wild type α-synuclein (17).

The PTD itself is a functional nuclear localization signal (NLS), and thereby, essentially accompanies the nuclear import of attached molecules (18, 19). This implies that every clinical application of PTD-mediated transduction may have to address the possibility of damage to nuclear genetic agents. Although PTDs are beginning to be applied in protein therapy, some weaknesses are still encountered, particularly due to the lack of specificity toward target proteins. Therefore, improving the specificity of PTDs is required.

Hawiger and colleagues reported that synthetic peptide SN-50, consisting of a membrane permeable motif and nuclear localization sequence, carried a functional cargo representing the nuclear localization sequence of NF-κB p50, and this fusion protein inhibited the nuclear translocation of NF-κB (20, 21).

Therefore, we have designed NLS and membrane translocation sequence (MTS) signal peptides, and assessed their transduction potential and subcellular localization after transduction. The NLS and MTS peptide fused proteins localized mainly in the nucleus and cytosol of cells, with enzymatic activities. Therefore, we suggest that NLS and MTS peptides can be effectively applied in the field of protein therapy.

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RESULTS AND DISCUSSION

Construction and purification of NLS-SOD and MTS-SOD fusion proteins

To generate a cell-permeable expression vector, as well as a NLS-SOD and MTS-SOD vector, a human SOD cDNA was

subcloned into the pET-15b plasmid that had been reconstructed to contain the NLS and MTS peptides. Thus, the formed NLS-SOD and MTS-SOD expression vector contained consecutive cDNA sequences encoding human SOD, NLS-SOD, and MTS-SOD peptides as well as six histidine residues at the amino-terminus (Fig. 1A). We also constructed the SOD expression vector to produce control SOD protein without the NLS and MTS transduction peptides (data not shown).

Following the induction of expression, the SOD fusion proteins were purified. The fusion proteins were expressed in *E. coli* and the clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. The fusion protein-containing fractions were combined and the salts were removed using a PD10 column. The crude cell extracts obtained from *E. coli* and the purified SOD fusion proteins were electrophoresed in 12% SDS-PAGE. As shown in Fig. 1B, the SOD fusion proteins were highly expressed, and the recombinant SOD fusion proteins were nearly homogenous and greater than 95% pure, as determined by SDS-PAGE analysis with Coomassie brilliant blue staining.

Transduction of NLS-SOD and MTS-SOD fusion proteins into HeLa cells

To evaluate the transduction ability of the NLS-SOD and MTS-SOD fusion proteins, we analyzed their transduction by adding them to HeLa culture medium at 3 μM for 60 min, and then analyzed the transduced protein levels by Western blotting. Intracellular concentrations of the transduced NLS-SOD and MTS-SOD fusion proteins in the cells were markedly increased after 60 min (Fig. 2A). The purified NLS-SOD and MTS-SOD fusion proteins efficiently transduced into the cells in time- and dose-dependent manners; however, they each showed different transduction patterns (data not shown). This slight difference in their transduction efficiencies may have depended on whether the target protein fused with the NLS and MTS peptides had a different conformation, polarity, or molecular shape.

The restoration of the authentic properties of transduced proteins in cells is a key aspect of applying protein transduction technology for therapeutic use. Therefore, we determined the dismutation activity of SOD in HeLa cells treated with NLS-SOD and MTS-SOD fusion proteins, as well as SOD protein as a control under the same conditions. The intracellular dismutation activity of SOD increased four times after treatment with 3 μM NLS-SOD and MTS-SOD fusion protein for 60 min, whereas the control SOD did not change SOD activity (Fig. 2B). The enzyme activities increased after treating with various concentrations of NLS-SOD and MTS-SOD fusion protein (data not shown). These results demonstrate that NLS-SOD and MTS-SOD fusion proteins can be transduced efficiently into cells, offering potential use as protein therapy.

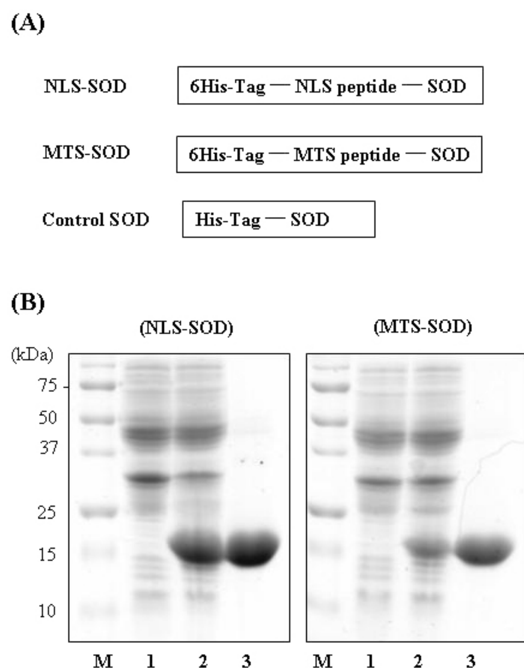

Fig. 1. Purification of NLS-SOD and MTS-SOD fusion proteins. A schematic representation of the NLS-SOD and MTS-SOD fusion proteins containing 6 His, NLS/MTS, and SOD coding sequences (A). The expressed and purified SOD fusion proteins were analyzed by 12% SDS-PAGE (B). Lanes are as follows: lane 1, non-induced; lane 2, induced; lane 3, purified.

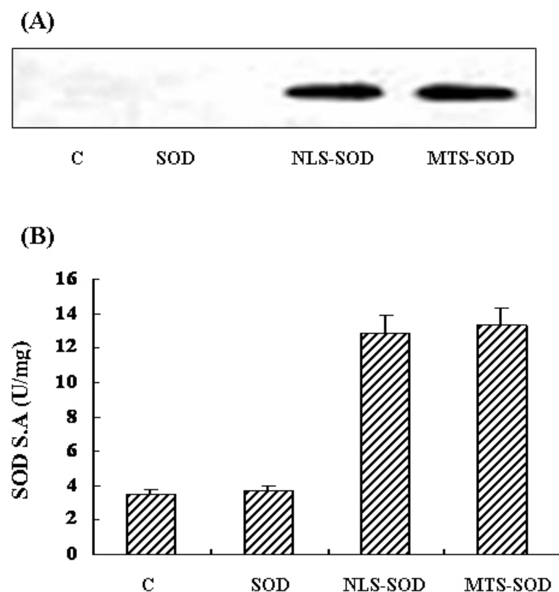

Fig. 2. Transduction of NLS-SOD and MTS-SOD fusion proteins into HeLa cells. Three μM of the SOD fusion proteins were added to each cultured media for 60 min. The transduced SOD fusion proteins were analyzed by Western blotting (A) and for specific enzyme activities (B).

Subcellular localization of NLS-SOD and MTS-SOD fusion proteins

The NLS-SOD and MTS-SOD fusion proteins were assessed by a fluorescence microscope, in an attempt to characterize their subcellular localization after transduction. In the cells treated with NLS-SOD, the majority of SOD fluorescent signals were detected in the nucleus, and were clearly separated from the MTS-SOD signals observed in the fluorescence microscopic image. As shown in Fig. 3A, the NLS-SOD and MTS-SOD fusion proteins were present in both the nucleus and cytosol.

To clarify further the subcellular localization of the transduced proteins into various cells (HeLa cells, glia cells, and astrocytes), nuclear and cytosolic fractions were prepared from cells transduced with the NLS-SOD and MTS-SOD fusion proteins, and then analyzed by Western blotting using anti-histidine, anti-actin, and anti-PARP antibodies. As shown in Fig. 3B, NLS-SOD and MTS-SOD fusion proteins were detected at similar intensities in the nuclei as well as cytoplasms of the transduced cells. At the present time, although many PTDs are beginning to be applied in protein therapy, some weaknesses are still encountered, particularly due to the lack of specificity toward target proteins. However, in this study, our results indicate that the subcellular localization of transduced fusion proteins was attributed to the intrinsic properties of NLS and MTS.

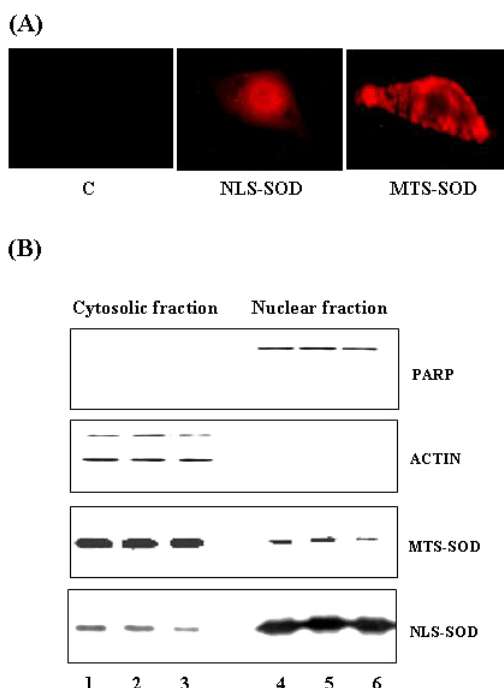

Fig. 3. Subcellular localization of NLS-SOD and MTS-SOD fusion proteins. Images of HeLa cells transduced with SOD fusion protein. The HeLa cells were treated with 3 μM of the SOD fusion proteins for 60 min, and then the cells were incubated with anti-rabbit polyhistidine (1:400) and Cy-3 conjugated antibody (1:1,000) for 60 min. The cells were washed twice with trypsin-EDTA and PBS and immediately observed by fluorescence microscopy (A). Analysis of the nuclear and cytosolic fractions of cells transduced with SOD fusion proteins. The nuclear and cytosolic extracts were prepared from tranduced cells and analyzed by Western blotting with anti-rabbit polyhistidine antibody. The membrane was stripped and re-probed with anti-actin and anti-PARP antibody (B). Lanes are as follows: lanes 1 and 4, HeLa cells; lanes 2 and 5, glia cells; lanes 3 and 6, astrocyte cells.

Effect of transduced NLS-SOD and MTS-SOD fusion proteins on the viability of cells under oxidative stress

To determine whether the transduced NLS-SOD and MTS-SOD fusion proteins could play biological roles in cells, we examined the effect of the SOD fusion proteins on cell viability after the administration of paraquat (methyl viologen). The viability of cells treated with paraquat increased when they were pretreated with 3 μM of the SOD fusion proteins. As shown in Fig. 4, when the cells were exposed to 5 mM paraquat without SOD fusion protein, only 36% of the cells were viable. By comparison, the viability of the cells pretreated with SOD fusion protein increased approximately 80%. These results indicate that the transduced NLS-SOD and MTS-SOD fusion proteins were definitely effective against the induction of superoxide anion by paraquat in HeLa cells.

Overall, the present experimental results demonstrate that NLS-SOD and MTS-SOD fusion proteins can be efficiently transduced into cells, and the delivered proteins exhibit a cellular protective function against oxidative stress. In particular, the NLS-SOD and MTS-SOD fusion proteins efficiently transduced into different subcellullar localizations, within the nucleus and cytosol, respectively. Although the details of the mechanism need to be further elucidated, our success in the var-

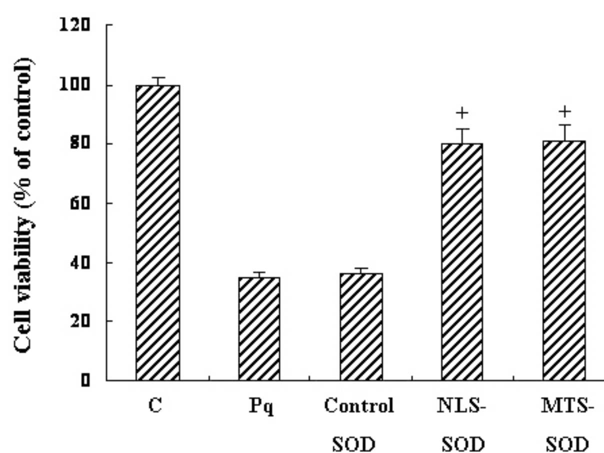

Fig. 4. Effects of transduced NLS and MTS-SOD fusion proteins on cell viability. Paraquat (Pq, 5 mM) was added to HeLa cells pretreated with 3 μM SOD fusion protein for 1 h. The Cell viabilities were estimated by a colorimetric assay using MTT. Each bar represents the mean ± S.E.M. obtained from five experiments.

ied localization of the anti-oxidant enzyme SOD may provide a useful tool in the field of protein therapy.

MATERIALS AND METHODS

Construction and purification of NLS-SOD and MTS-SOD fusion proteins

Synthetic cell-permeable SN50 peptide, which is used to study other intracellular processes involving proteins with functionally distinct domains, has a membrane translocation sequence (MTS; AAVALLPAVLLALLAP) and a nuclear localization sequence (NLS; VQRKRQKLMP). NLS-SOD and MTS-SOD expression vectors were constructed to express the NLS and MTS peptides as a fusion with human Cu, Zn-SOD. The synthesized oligonucleotides were ligated into a *Nde*I-*Xho*I-digested pET-15b vector. Next, based on the cDNA sequence of human SOD, two primers were synthesized (7, 22). The PCR reaction was performed and the PCR products were excised with *Xho*I and *Bam*HI, eluted (Invitek, Berlin, Germany), ligated into TA-cloning vector (Promega, Madison, WI, USA) and the NLS and MTS vectors using T4 DNA ligase (Takaka, Otsu, Shiga, Japan), and finally cloned in *E. coli* DH5α cells. NLS-SOD and MTS-SOD fusion proteins were generated when the human SOD gene was fused with an NLS or MTS peptide in a bacterial expression vector, in order to produce genetic in-frame NLS-SOD and MTS-SOD fusion proteins.

To produce the NLS-SOD and MTS-SOD fusion proteins, each plasmid was transformed into *E. coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of LB media at 37°C to a D_{600} value of 0.5 ~ 1.0, and induced with 0.5 mM IPTG at 30°C for 12 h. The harvested cells were lysed by sonication at 4°C in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea, and the recombinant NLS-SOD and MTS-SOD that formed was purified. Briefly, clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA). After the column was washed with 10 volumes of a binding buffer and six volumes of a wash buffer (35 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9), the fusion proteins were eluted using an eluting buffer (0.5 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fusion protein-containing fractions were combined and the salts were removed using a PD-10 column (Amersham, Braunschweig, Germany). The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (23).

Transduction of NLS-SOD and MTS-SOD fusion proteins into HeLa cells

For the transduction of the NLS-SOD and MTS-SOD fusion proteins, HeLa cells were grown to confluence on a 6-well plate. Then, each culture medium was replaced with 1 ml of fresh solution. After the HeLa cells were treated with 3 μM of NLS-SOD and MTS-SOD fusion protein for 1 h, the cells were treated with trypsin-EDTA (Gibco, Grand Island, NY, USA) and washed with phosphate-buffered saline (PBS). The cells were harvested to prepare the cell extracts utilized in the SOD enzyme assay and Western blot analysis.

Enzymatic assay of SOD

The dismutation activities of SOD were measured by monitoring the inhibition of ferricytochrome *c* reduction by the xanthine/xanthine oxidase reaction (24, 25). The assay was performed in 3 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA in a cuvette at 25°C. The reaction mixture contained 10 μM cytochrome *c*, 50 μM xanthine, and sufficient xanthine oxidase to produce a rate of reduction of cytochrome *c* at 550 nm of 0.025 absorbance units per min. Under these defined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome *c* by 50% (to a rate of 0.0125 absorbance units per min) is defined as 1 unit of activity.

Immunofluorescence analysis

An immunofluorescene assay was performed using conjugated Cy-3 antibodies. Briefly, HeLa cells were grown on glass coverslips and treated with NLS-SOD and MTS-SOD fusion protein. Following incubation for 1 h at 37°C, the cells were washed twice with trypsin-EDTA and PBS, and then fixed in 4% paraformaldehyde in 0.5 ml of PBS for 10 min at room temperature. The cells were washed with PBS and then incubated with a polyhistidine antibody, followed by incubation with Cy-3 antibody (1:1,000) in PBS for 1 h. The distribution of fluorescence was analyzed with on a fluorescence microscope (Carl Zeiss, EL-Einsatz, Germany).

Subcellular fractionation of the transduced cells

The nuclear and cytosolic fractions were prepared as previously described (26, 27). The transduced HeLa cells were washed with PBS, acid-washed with 0.2 M glycine-HCl (pH 2.2), and trypsinized for 10 min at 37°C. The cells were harvested after washing with cold PBS and pelleted. They were then resuspended in 1 ml of NP-40 buffer by gentle pipetting, and incubated on ice for 10 min. Next, the cells were spun through a sucrose cushion at 1,000 *g* for 10 min and the cytosolic fractions were collected from the supernatants. The pellets were washed with 1 ml of NP-40 buffer to completely remove the cytosolic fractions. The nuclei were lysed in a lysis buffer (50 ml Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1% Triton X-100). The resulting nuclear and cytosolic lysates were analysed by Western blotting.

Western blot analysis

For Western blot analysis, 15 μg of the protein from each whole cell lysate was run on a 12% SDS-PAGE. The transduced SOD fusion proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05%

Tween-20 (TBST) for 2 h, and was then incubated for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ, USA). The same membrane was stripped and re-probed with an anti-actin antibody (cytosolic marker; Oncogene) or an anti-poly (ADP-riobse) polymerase (PARP) antibody (nuclear marker; Biomol. Plymouth Meeting, PA).

MTT assay

The biological activities of the transduced NLS-SOD and MTS-SOD fusion proteins were assessed by measuring the cell viability of HeLa cells treated with paraquat (methyl viologen; Sigma Chemical Co. St. Louis, MO, USA), which is a well-known intracellular superoxide anion generator (28). The cells were seeded in 24-well plates at 70% confluence and pretreated with 3 μM of control SOD and NLS-SOD and MTS-SOD for 1 h, respectively. Then 5 mM of paraquat was added to the culture medium for 12 h. The cell viability was estimated by a colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide).

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