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Tat-mediated Protein Transduction of Human Brain Pyridoxine-5-P Oxidase into PC12 Cells

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Pyridoxine-5-P oxidase catalyses the terminal step in the biosynthesis of pyridoxal-5-P, the biologically active form of vitamin B_6 Which acts as an essential cofactor. Here, a human brain pyridoxine-5-P oxidase gene was fused with a gene fragment encoding the HIV-1 Tat protein transduction domain (RKKRRQRRR) in a bacterial expression vector to produce a genetic in-frame Tat-pyridoxine-5-P oxidase fusion protein. Expressed and purified Tat-pyridoxine-5-P oxidase fusion protein transduced efficiently into PC12 cells in a time- and dose-dependent manner when added exogenously to culture media. Once inside the cells, the transduced Tat-pyridoxine-5-P oxidase protein showed catalytic activity and was stable for 48 h. Moreover, the formation of pyridoxal-5-P was increased by adding exogenous Tat-pyridoxine-5-P oxidase to media pre-treated with the vitamin B_6 precursor pyridoxine. In addition, the intracellular concentration of pyridoxal-5-P was markedly increased when Tat-pyridoxal kinase was transduced together with Tat-pyridoxine-5-P oxidase into cells. These results suggest that the transduction of Tat-pyridoxine-5-P oxidase fusion protein presents a means of regulating the level of pyridoxal-5-P and of replenishing this enzyme in various neurological disorders related to vitamin B_6.

Keywords: HIV-1 Tat, Protein transduction, Pyridoxine-5-P oxidase, Pyridoxal-5-P, Protein therapy.

Introduction

Pyridoxal-5-P, the biologically active form of vitamin B_6, serves as a cofactor and is required by numerous enzymes that catalyze transamination and decarboxylation reactions (McCormick *et al.*, 1961; Snell, 1990). Mammalian pyridoxal-5-P-containing enzymes are involved in the synthesis of various neurotransmitters, such as, dopamine, serotonin, γ-aminoguatrate (GABA), and modulate steroid-receptor interactions and regulate immune function (Dakshinamurti *et al.*, 1990; Allgood *et al.*, 1993).

The metabolism of pyridoxal-5-P has been studied extensively in normal tissues by several laboratories (Kwok and Churchich, 1980; Meisler and Thanassi, 1980; Choi *et al.*, 1987; Bahn *et al.*, 2002; Kang *et al.*, 2002a). Pyridoxal-5-P is formed in mammalian cells by the phosphorylation of pyridoxal by pyridoxal kinase or by the oxidation of pyridoxine-5-P or pyridoxamine-5-P by a cytosolic enzyme pyridoxine-5-P oxidase (EC 1.4.3.5). Mammalian pyridoxine-5-P oxidase is a dimer composed to two identical subunits of about 30 kDa each. Its flavin mononucleotide (FMN) group acts as a coenzyme and is absolutely required for its catalytic activity. In prokaryotic organisms, pyridoxine-5-P oxidase is involved in the terminal step of the *de novo* biosynthesis of pyridoxal-5-P, which in eukaryotic cells is a part of a salvage pathway used for the reutilization of pyridoxine-5-P. Although it is known that pyridoxine-5-P is the preferred substrate of the oxidase, pyridoxine-5-P oxidase also catalyzes the oxidation of secondary amines (Choi *et al.*, 1983).

Pyridoxine-5-P oxidase is well distributed in mammals and is expressed at high activity levels in a wide range of tissues, which include liver, kidney, and brain, and at relatively lower activity levels in heart, skeletal muscle, pancreas, and bone

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activity levels in heart, skeletal muscle, pancreas, and bone

marrow. The different oxidase activity levels found in tissues have led to the establishment of a complicated network for pyridoxal-5-P (Kazarinoff and McCormick, 1975; Black *et al.*, 1977; Churchich, 1984; Lam and Winkler, 1992; Zhao and Winkler, 1995). Pyridoxine-5-P oxidase is of particular interest because abnormalities in vitamin B_6 metabolism are known to be related to neuronal disorders. Several lines of evidence indicate that convulsive seizures may be induced by vitamin B_6 deficiency (Gospe *et al.*, 1994; Glenn *et al.*, 1995; Waymire *et al.*, 1995), and although no obvious connections have been elucidated, pyridoxine-5-P oxidase is a clear candidate target in these disorders.

Several small regions of proteins called protein transduction domains (PTDs) have been developed to allow the delivery of exogenous proteins into living cells. These include carrier peptides derived from HIV-1 Tat protein, *Drosophila* Antennapedia (Antp) protein, and herpes simplex virus VP22 protein (Frankel and Pabo, 1988; Derrosi *et al.*, 1994; Elliott and O'Hare, 1997). By using this protein transduction technology, we found that a genetic in-frame Tat-green fluorescent fusion protein (Tat-GFP), Tat-Cu,Zn superoxide dismutase (Tat-SOD), and Tat-catalase fusion proteins were all efficiently transduced into mammalian cells and skin (Kwon *et al.*, 2000; Han *et al.*, 2000; 2001; Jin *et al.*, 2001; Eum *et al.*, 2002; Park *et al.*, 2002a; 2002b). More recently, we reported that transduced Tat-SOD significantly protected pancreatic β-cells from oxidative stress-induced destruction, and improved the diabetic status of streptozotocin-induced diabetic mice. In addition, PEP-1-SOD fusion proteins were found to prevent neuronal cell death in the hippocampus caused by transient forebrain ischemia (Eum *et al.*, 2004a; 2004b).

In this paper, we describe the transduction of full length human brain Tat-pyridoxine-5-P oxidase fusion protein into PC12 cells and the resulting biological activity. Our results suggest that this transduction of Tat-pyridoxine-5-P oxidase fusion protein offers the possibility of the development of therapies for various disorders related to Tat pyridoxine-5-P oxidase and vitamin B_6.

Materials and Methods

Materials. Ni^{2+}-nitrilotriacetic acid Sepharose superflow was purchased from Qiagen; isopropyl-β-D-thiogalactoside (IPTG) from Duchefa Co; fetal bovine serum (FBS), RPMI1640, and penicillin-streptomycin antibiotics from Gibco BRL; and pyridoxine, ATP, and goat anti rabbit immunoglobulins from Sigma. A human pyridoxine-5-P oxidase cDNA fragment was isolated using PCR from the human brain cDNA library. Rabbit anti-histidine polyclonal antibody was purchased from Santa Cruz Biotechnology, and a monoclonal antibody raised against pyridoxine-5-P oxidase was produced as previously described (Bahn *et al.*, 2002). All other chemicals and reagents were of the highest analytical grade available.

Construction, expression, and purification of Tat-pyridoxine-5-P oxidase. Construction of the various pTat fusion protein plasmids with the HIV-1 Tat protein transduction domain (PTD; amino acids $_{49-57}$) were performed as previously described (Kwon *et al.*, 2000; Jin *et al.*, 2001; Eum *et al.*, 2004a; Kim *et al.*, 2005b). Briefly, the pyridoxine-5-P oxidase gene was amplified by PCR using the following two primers; the sense primer was 5'-ACACTCGAGAT GGAGGAGGAGTGCCGGGTGCTC-3' (containing an *Xho*I restriction site), and the antisense primer was 5'-TGTGGATCCTCACAGCAC CGTGGCCTGGACGAC-3' (containing a *Bam*HI restriction site). PCR products were purified and cloned into TA cloning vector. After digesting with *Xho*I and *Bam*HI, the Tat-pyridoxine-5-P oxidase gene was ligated into the expression vector, pET-15b, in frame together with a six histidine open-reading frame to generate the expression vector. Tat-pyridoxine-5-P oxidase and pyridoxine-5-P oxidase in pET-15b vector were expressed in *E. coli* BL21 (DE3). The host *E. coli* BL21 (DE3) was transformed with plasmids encoding pPyridoxine-5-P oxidase or pTat-pyridoxine-5-P oxidase, and transformants were selected on an LB plate containing ampicillin. The selected colonies were cultured in LB medium containing ampicillin at 37°C with shaking at 250 rpm. After allowing cell growth until $O.D_{600}$ = 0.5~0.6, protein expression was induced by adding IPTG to a final concentration of 1 mM and incubation was continued for 3~4 hr. Cells were harvested and a 5 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea was added and sonicated. After centrifugation, supernatants containing Tat-pyridoxine-5-P oxidase were immediately loaded on a 2.0 ml Ni^{2+}-nitrilotriacetic acid Sepharose column, and after washing the column with 10 volumes of binding buffer and 6 volumes of washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), the fusion protein was eluted using an elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The salts in the purified fractions were removed by PD10 column chromatography (Kang *et al.*, 2004), and protein concentrations were estimated using Bradford's method using bovine serum albumin as a standard (Bradford, 1976).

Transduction of Tat-pyridoxine-5-P oxidase into cultured PC12 cells. PC12 cells were grown in RPMI1640 containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, heat-inactivated 10% horse serum, heat-inactivated 5% fetal bovine serum (FBS), and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C in humidified 95% air-5% CO_2. To transduce Tat-pyridoxine-5-P oxidase, PC12 cells were grown to confluence on a 6-well plate, and then the medium was replaced with 1ml of fresh solution. The cells were then treated with various concentrations of Tat-pyridoxine-5-P oxidase for 1 h, followed by trypsin-EDTA, and washed with phosphate-buffered saline (PBS). They were then harvested and cell extracts were prepared for pyridoxine-5-P oxidase enzyme assays and Western blot analysis.

Fluorescence microscopy. To directly detect fluorescein-labeled protein, purified Tat-pyridoxine-5-P oxidase was labeled using an EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (PIERCE). FITC labeling was carried out according to the manufacturer's instructions. PC12 cells were grown on glass

coverslips and treated with 2 μM Tat-pyridoxine-5-P oxidase fusion protein for 1 h at 37°C, and then washed with trypsin-EDTA treated PBS. They were fixed in 4% paraformaldehyde for 10 min at room temperature, and fluorescence distributions were analyzed using a Zeiss Axiophot fluorescence microscope.

Formation of pyridoxal-5-P in cells transduced Tat-pyridoxine-5-P oxidase. PC12 cells were plated into 6 well trays at 70% confluence, and allowed to attach cells per well. They were then pre-treated with 1~2 mM of the vitamin B_6 precursor pyridoxine for 3 h and washed three times with PBS to remove free pyridoxine. 2 μM of Tat-pyridoxine-5-P oxidase was then transduced into the cells for 1 h. The concentrations of free pyridoxal-5-P in cells were measured using a spectroscopic method, as previously described (Choi *et al.*, 1992; Kim *et al.*, 2005b).

Western blot analysis. Proteins in cell extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated for 1h in block solution [5% nonfat dry milk in Tris-buffered saline (TBS)], washed three times with TBS containing Tween 20, and incubated with rabbit anti-histidine polyclonal antibody (dilution 1 : 500) and anti-pyridoxine-5-P oxidase monoclonal antibody for 1 h at room temperature. After washing three times with TBS containing Tween 20, they were incubated with goat anti-rabbit immunoglobulins (Sigma, dilution 1 : 10,000) for 1 h, and bound antibodies were visualized by enhanced chemiluminescence, according to the manufacturer's instructions (ECL; Amersham) (Kim *et al.*, 2003).

Pyridoxine-5-P oxidase enzyme assays. Assays were performed as described by Kwon *et al.* (1991). Briefly, pyridoxine-5-P oxidase activities were measured at pH 8.4 in 0.1 M Tris-HCl. The formation of pyridoxal-5-P was measured by following increases in absorbance at 388 nm for at least 3 min. At this wavelength, pyridoxal-5-P is known to have a molecular extinction coefficient of 4,900 cm^{-1} M^{-1} at pH 7. The standard assay mixture contained 0.5 mM pyridoxal, 0.5 mM ATP, and 0.1 mM $ZnCl_2$ in a 70 mM potassium phosphate buffer (pH 6.0). One unit of activity was defined as the amount of enzyme required to form 1 nmol of pyridoxal-5-P/min at 37°C.

Results

The construction, expression, and purification of Tat-pyridoxine-5-P oxidase fusion protein. Tat-pyridoxine-5-P oxidase expression vector contained a consecutive cDNA sequence encoding human pyridoxine-5-P oxidase, the Tat-protein transduction domain, and 6 histidine residues at the Tat-pyridoxine-5-P oxidase amino-terminus (Fig. 1A). We also constructed a pyridoxine-5-P oxidase expression vector to produce control pyridoxine-5-P oxidase protein without a HIV-1 Tat protein transduction domain (data not shown). Bacterial cells induced with IPTG were lysed at 4°C in PBS buffer. Crude cell extracts obtained from *E. coli* were electrophoresed in 12% SDS-PAGE. Fig. 1B shows protein bands visualized by Coomassie brilliant blue staining. Tat-

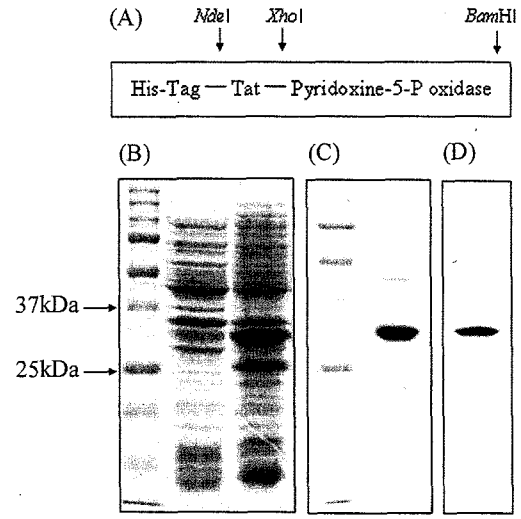

Fig. 1. Diagram of expressed Tat-pyridoxine-5-P oxidase fusion protein. The coding frame of human brain pyridoxine-5-P oxidase is presented with a hexahistidine and HIV-1 Tat protein transduction domain (PTD; RKKRRQRRR) (A). Expression and purification of Tat-pyridoxine-5-P oxidase in *E. coli*. Protein extracts of cells (B) and purified fusion proteins (C) were analyzed by 12% SDS-PAGE and subjected to Western blot analysis using rabbit anti-histidine polyclonal antibody (D).

pyridoxine-5-P oxidase protein found to be expressed at a high level and was a major component of the total cellular soluble proteins. Tat-pyridoxine-5-P oxidase fusion proteins were purified, as confirmed by SDS-PAGE (Fig. 1C), which showed a molecular mass of approximately 31 kDa. The purified products were further confirmed by Western blotting using a rabbit anti-histidine polyclonal antibody (dilution 1 : 500) (Fig. 1D).

Transduction of Tat-pyridoxine-5-P oxidase fusion protein into PC12 cells. The intracellular delivery of Tat-pyridoxine-5-P oxidase into PC12 cells was confirmed by direct fluorescence. To exclude the possibility that cell fixation with paraformaldehyde may affect Tat-pyridoxine-5-P oxidase transduction by direct fluorescence, we transduced FITC-labeled Tat-pyridoxine-5-P oxidase protein into non-fixed or fixed PC12 cells. As shown in Fig. 2, immunofluorescence staining using a rabbit anti-histidine polyclonal antibody revealed that Tat-pyridoxine-5-P oxidase fusion protein transduced into the cells, whereas fluorescence signals were absent in cells not treated with Tat-pyridoxine-5-P oxidase (Fig. 2A). In addition, fluorescence signals of non-fixed cells were similar to those of fixed cells (Fig. 2B and 2C). These results indicate that cell fixation is not required for Tat-pyridoxine-5-P oxidase transduction.

To evaluate the transduction ability of Tat-pyridoxine-5-P oxidase, 2 μM of Tat-pyridoxine-5-P oxidase fusion proteins, purified under denaturing conditions, were added to PC12 cells in culture for various times and concentrations. As

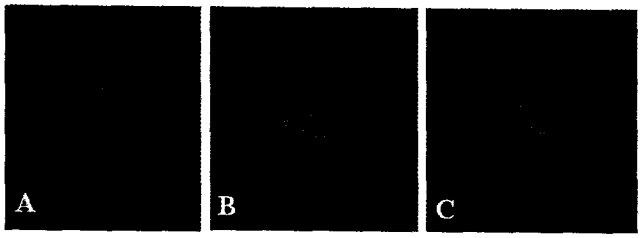

Fig. 2. The intracellular distribution of Tat-pyridoxine-5-P oxidase fusion proteins in PC 12 cells fixed or not fixed with paraformaldehyde. FITC-labeled Tat-pyridoxine-5-P oxidase fusion proteins (2 μM) were transduced into PC12 cells for 1 h, cells were then washed once with trypsine, and twice with PBS, and immediately observed under a fluorescence microscope. Control cells not treated with Tat-pyridoxine-5-P oxidase (A), non-fixed cells treated with Tat-pyridoxine-5-P oxidase (B), and fixed cells treated with Tat-pyridoxine-5-P oxidase (C).

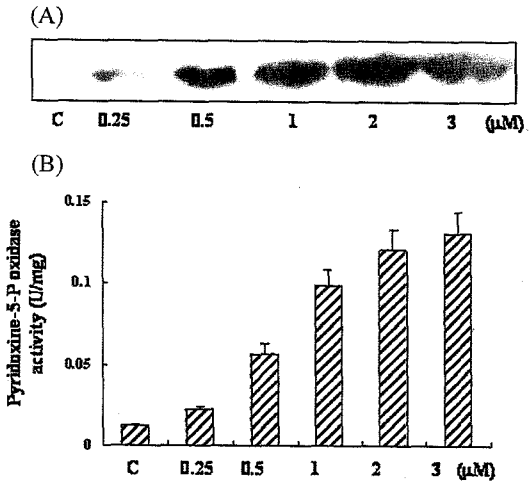

Fig. 3. Dose-dependent transduction of Tat-pyridoxine-5-P oxidase into PC12 cells. 0.25~3 μM of denatured Tat-pyridoxine-5-P oxidase was added to culture media for 1h, and cell lysates were analyzed by Western blotting (A) and for enzyme activities (B).

shown in Fig. 3A, Tat-pyridoxine-5-P oxidase fusion proteins transduced into PC12 cells in a dose dependent manner. Denatured Tat-pyridoxine-5-P oxidase proteins were added to PC12 cells in culture at various concentrations (0.25~3 μM) for 1 h, and the levels of transduction and pyridoxine-5-P oxidase enzyme activity were analyzed by Western blotting and by determining catalytic activity, respectively. The catalytic activities of Tat-pyridoxine-5-P oxidase in transduced cells were 3-8 folds higher than in untransduced cells and fusion protein levels in media were concomitantly increased (Fig. 3B). The time dependency of Tat-pyridoxine-5-P oxidase fusion protein transduction was further analyzed. 2 μM of Tat-pyridoxine-5-P oxidase protein was added to PC12 cells in culture for various times (10~60 min), and levels of transduced proteins were measured by Western blotting. As shown in Fig. 4A, the intracellular concentration of Tat-pyridoxine-5-P oxidase transduced into cultured cells gradually

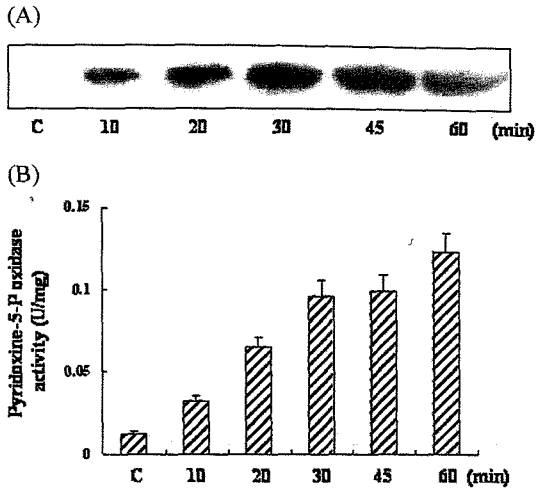

Fig. 4. Time-dependent transduction of Tat-pyridoxine-5-P oxidase into PC12 cells. 2 μM Tat-pyridoxine-5-P oxidase was added to culture media for 10~60 min. Lysates were analyzed by Western blotting (A) and for enzyme activities (B).

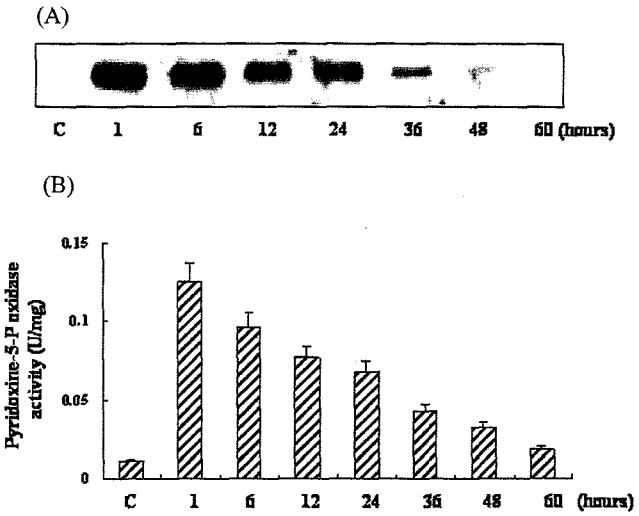

Fig. 5. The stability of Tat-pyridoxine-5-P oxidase transduced into PC12 cells. Cells pretreated with 2 μM Tat-pyridoxine-5-P oxidase were incubated for 1~60 h. Levels of Tat-pyridoxine-5-P oxidase and its activities were determined by Western blotting (A) and by using enzyme assays (B).

increased, and the catalytic activity of the transduced enzyme increased in a time-dependent manner (Fig. 4B). The intracellular stabilities and enzymatic activities of transduced Tat-pyridoxine-5-P oxidase in PC12 cells are shown in Fig. 5. An apparent degradation of transduced Tat-pyridoxine-5-P oxidase. PC12 cells was observed to occur as a function of incubation time. However, significant levels of the transduced protein and its enzyme activity persisted at 48 h after transduction.

Formation of pyridoxal-5-P by transduced Tat-pyridoxine-5-P oxidase. To confirm that the transduced Tat-pyridoxine-5-

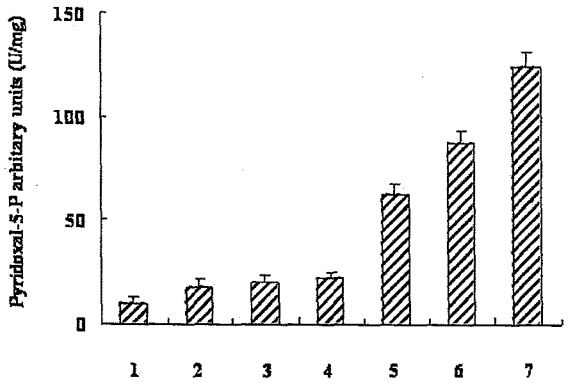

Fig. 6. Pyridoxal-5-P production by transduced Tat-pyridoxine-5-P oxidase in PC 12 cells pre-treated with vitamin B_6 precursor pyridoxine. Cells pre-treated with 1~2 mM pyridoxine were incubated for 3 h and 2 μM Tat-pyridoxine-5-P oxidase and Tat-pyridoxal kinase fusion proteins were added for 1 h. The production of pyridoxal-5-P in PC12 cells was analyzed spectroscopically. The bars are as follow: lane 1, PC12 cells only; lane 2, 1 mM pyridoxine only; lane 3, Tat-pyridoxine-5-P oxidase; lane 4, Tat-pyridoxal kinase; lane 5, Tat-pyridoxine-5-P oxidase + 1 mM pyridoxine; lane 6, Tat-pyridoxine-5-P oxidase + 2 mM pyridoxine; lane 7, Tat-pyridoxine-5-P oxidase + Tat-pyridoxal kinase + 2 mM pyridoxine.

P oxidase was biologically active in PC12 cells, we tested its catalytic activity after adding exogenous pyridoxine (vitamin B_6) to cells. When cells pre-treated with Tat-pyridoxine-5-P oxidase (2 μM) were exposed to 1~2 mM pyridoxine, pyridoxal-5-P formation increased significantly by 5~7-fold (Fig. 6). Moreover, the combined supply of Tat-pyridoxine-5-P oxidase and Tat-pyridoxal kinase into cells, increased the intracellular concentration of pyridoxal-5-P more so than Tat-pyridoxine-5-P oxidase alone. This result suggests that transduced Tat-pyridoxine-5-P oxidase fusion proteins convert vitamin B_6 into active cofactor from pyridoxal-5-P.

Discussion

Pyridoxal-5-P is a biologically active form of vitamin B_6, and acts as an essential metabolic coenzyme in mammals. Moreover, mammals cannot synthesize pyridoxal-5-P *de novo,* and thus, require dietary precursors like pyridoxine, pyridoxal, or pyridoxamine. However, once inside cells, pyridoxal-5-P is formed from these precursors by two enzymes, pyridoxine-5-P oxidase and pyridoxal kinase. In the mammalian nervous system, several neurotransmitters, e. g., dopamine, norepinephrine, serotonin, and γ-aminobutyric acid (GABA) are synthesized by pyridoxal-5-P-dependent enzymes (Dakshinamurti *et al.*, 1990). Since several lines of evidence indicate that convulsive seizure is associated with vitamin B_6 deficiency, and that the pyridoxal-5-P synthesizing enzymes, pyridoxine-5-P oxidase and pyridoxal kinase, are involved in this disease (Kang *et al.*, 2002a; 2002b), it is worthwhile considering the therapeutic use of these enzymes. Therefore, to replenish pyridoxine-5-P oxidase levels in patients suffering from convulsive seizures, we suggest that a developmental approach be investigated involving Tat-pyridoxine-5-P oxidase fusion protein transduction into brain cells or tissues.

HIV-1 Tat PTD allows highly efficient protein transduction through the plasma membrane. And, although the mechanism of transduction is unclear, Tat PTD fusion protein transduction has become a pharmacologic developmental issue. Our previous studies have shown that various Tat PTD fusion proteins are efficiently transduced into various mammalian cells, including HeLa, RINm5F, primary islet, and PC12 cells, fibroblasts, and astrocytes (Kwon *et al.*, 2000; Jin *et al.*, 2001; Park *et al.*, 2002b; Eum *et al.*, 2004a; Kim *et al.*, 2005a) and into the brain, heart, kidney, lung, pancreas, spleen, liver, and skin in mouse (Kim *et al.*, 2005a). In addition, antioxidant enzymes, such as, Tat-SOD and Tat-catalase fusion proteins, when applied topically, may have therapeutic potential against various skin disorders like skin inflammatory disease, which is mediated by reactive oxygen species (Jin *et al.*, 2001; Park *et al.*, 2002b). Our results are consistent with those of several other studies, in which Tat PTD fusion proteins containing full-length protein, were found to enter any cell type and to be delivered to all tissues, and that they even cross the blood-brain barrier (Schwarze *et al*, 2000; Wadia and Dowdy, 2002; Kabouridis, 2003).

Recently, by using "protein transduction technology" a wide range of proteins have been successfully used to study intracellular functions in cells and tissues (Wadia and Dowdy, 2002). However, the intracellular protein transduction properties of Tat fusion peptides were questioned by Richard and colleagues (Richard *et al.*, 2003). They suggested that Tat fusion proteins strongly bind to the cell membrane in an endocytic-dependent fashion, and also demonstrated that cell fixation leads to the artificial redistribution of cell-penetrating peptides (CPP) into the nucleus.

However, in the present study, no difference was found in the fluorescence distributions of transduced Tat-pyridoxine-5-P oxidase in non-fixed and fixed cells, which demonstrates that cell fixation with paraformaldehyde is not required for Tat-pyridoxine-5-P oxidase transduction. Similar observations have been reported, where protein transduction artifacts were not found to occur with paraformaldehyde fixation (Brewis *et al.*, 2000; Cashman *et al.*, 2003; Eum *et al.*, 2004a; 2004b). In addition, our recent studies show that the transduction efficiency of Tat fusion protein is not affected by long-term photodynamic effects, and that artificial membrane rupture may not occur during the transduction process (Kim *et al.*, 2005b).

In the present study, we examined the ability of Tat-pyridoxine-5-P oxidase to transduce into PC12 cells. Levels of transduction were analyzed by Western blotting, and Tat-pyridoxine-5-P oxidase proteins were found to enter into PC12 cells in a time- and dose-dependent manner. Although the exact mechanism of transduction is still unclear, Derrosi *et al.* (1994) demonstrated that the Antennapedia homeodomain

(Antp), another protein transduction domain in Drosophila, is internalized by a receptor- and transporter-independent mechanism. In general, protein transduction using PTD Tat fusion proteins was found to require fusion protein denaturation before delivery, which increases the accessibility of Tat PTD domain. After translocation of Tat-protein through the membrane in an unfolded state, members of the HSP90 protein family refold the target protein within the cell into its active conformation. Thus, the biological activity of the transduced protein was found to be dependent on the refolding efficacy of HSP90 (Schwarze *et al.*, 2000). However, the intracellular refolding mechanisms of transduced proteins require further investigation.

The restoration of the authentic properties of transduced proteins in cells is a key aspect of the therapeutic applications of protein transduction technology. Therefore, in the present study, we determined the enzyme activities of pyridoxine-5-P oxidase in cells treated with Tat-pyridoxine-5-P oxidase. The enzyme activities of pyridoxine-5-P oxidase in cultured PC12 cells were found to increase dose- and time-dependently, and increased about 2~10 fold after treating cells with various concentrations of denatured Tat-pyridoxine-5-P oxidase. Thus, although Tat-pyridoxine-5-P was found not to be transduced in PC12 cells under non-denaturing conditions (data not shown), the above results indicate that protein unfolding is required for the efficient transduction of Tat-pyridoxine-5-P oxidase into PC12 cells, and that denatured Tat-pyridoxine-5-P oxidase in PC12 cells is correctly refolded by a molecular chaperone or by some other spontaneous process. However, the intracellular refolding mechanisms of transduced proteins remain to be elucidated.

Our examination of the transduction kinetics involved showed that it look about an hour to achieve maximum Tat-pyridoxine-5-P oxidase transduction. We previously reported that Tat-superoxide dismutase and PEP-1-superoxide dismutase are rapidly transduced into HeLa cells and astrocytes within one hour (Kwon *et al.*, 2000; Eum *et al.*, 2004b), and that it took 2 h to saturate Tat-catalase transduction (Jin *et al.*, 2001). However, Tat-β-galactosidase fusion protein was found to transduce rapidly into HepG2 cells, and approached its maximum intracellular concentration in less than 15 min (Schwartze *et al.*, 1999). In terms of therapeutic applications the stability of transduced proteins in cells is of importance. In the present study, transduced Tat-pyridoxine-5-P oxidase enzyme activity retained significant activity at 48 hr post-transduction, and it is worth noting that transduced Tat-superoxide dismutase was stable for 24 hr (Kwon *et al.*, 2000; Eum *et al.*, 2004a) and Tat-catalase for 60 hr (Jin *et al.*, 2001). These different transduction kinetic patterns and cell stabilities might be derived from properties, such as, unfolding degree, polarity, and protein shape.

In the present study, it was also found that transduced Tat-pyridoxine-5-P oxidase protein increase the formation of pyridoxal-5-P when the vitamin B_6 precursor pyridoxine was added to PC12 cells in culture. Under physiological conditions, the formation of pyridoxal-5-P depends upon the catalytic functions of two cytosolic enzymes, i.e., pyridoxal kinase and pyridoxine-5-P oxidase. The kinase catalyzes the formation of phosphorylated vitamins, whereas the oxidase catalyzes the oxidation of pyridoxine-5-P and pyridoxamine-5-P to pyridoxal-5-P. In brain tissues, the specific activities of pyridoxal kinase and pyridoxine-5-P oxidase are 71 and 90 nmole min^{-1} mg^{-1}, respectively (Kwok and Churchich, 1979; Churchich, 1984). Recently, we reported that transduced Tat-pyridoxal kinase increased the intracellular concentration of pyridoxal-5-P (Kim *et al.*, 2005b). Therefore, in the present study, we investigated whether pyridoxal-5-P formation is increased synergistically when Tat-pyridoxine-5-P oxidase and Tat-pyridoxal kinase are co-transduced, and it was found that the intracellular concentration of pyridoxal-5-P was significantly more increased by co-transduction than by transduction by either alone. This finding suggests that Tat-pyridoxine-5-P oxidase and Tat-pyridoxal kinase fusion proteins convert vitamin B_6 pyridoxine into the active cofactor form pyridoxal-5-P; moreover, it suggests that co-transduction with these two enzymes is likely to provide a valuable insight of the role of pyridoxal kinase and pyridoxine-5-P oxidase in the control of vitamin B_6 metabolism.

Although the gene and protein technologies have been widely investigated, many unanswered questions remain about the possible limitations of PTD technology and gene therapy *in vivo*. One major disadvantage of gene and protein therapy is its lack of targeting specificity. Therefore, for each therapeutic case, it is important to determine whether PTD-chimera or therapeutic genes have beneficial effects on diseased cells and whether they have adverse effects on healthy tissues. Nevertheless, we consider that PTD technology has greater benefits than gene therapy because it allows intracellular protein levels to be controlled directly.

In summary, the present study demonstrates that exogenous human pyridoxine-5-P oxidase fused with Tat basic domain residues, can be directly transduced into cultured PC12 cells, and that the resulting enzymatically active pyridoxine-5-P oxidase converts pyridoxine-5-P and pyridoxamine-5-P to pyridoxal-5-P. Thus, we conclude that the co-transduction of pyridoxine-5-P oxidase fusion proteins with Tat-pyridoxal kinase may offer a therapeutic tool for various disorders related to vitamin B_6 metabolism.

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