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Human brain pyridoxal-5'-phosphate phosphatase (PLPP): protein transduction of PEP-1-PLPP into PC12 cells

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Pyridoxal-5'-phosphate phosphatase (PLPP) catalyzes the dephosphorylation of pyridoxal-5'-phosphate (PLP). A human brain PLPP gene was fused with a PEP-1 peptide and produced a genetic in-frame PEP-1-PLPP fusion protein. The purified PEP-1-PLPP fusion protein was efficiently transduced into PC12 cells in a time- and dose-dependent manner when added exogenously to culture media. Once inside the cells, the transduced PEP-1-PLPP fusion protein was stable for 36 h. The concentration of PLP was markedly decreased by the addition of exogenous PEP-1-PLPP to media pretreated with the vitamin B_6 precursors; pyridoxine, pyridoxal kinase and pyridoxine-5'-phosphate oxidase into cells. The results suggest that the transduction of the PEP-1-PLPP fusion protein can be one mode of PLP level regulation, and to replenish this enzyme in the various neurological disorders related to vitamin B_6. [BMB reports 2008; 41(5): 408-413]

INTRODUCTION

Pyridoxal-5'-phosphate (PLP) is the coenzymatically active form of vitamin B_6 and plays an important role in maintaining the biochemical homeostasis of the body (1, 2). The major pathways of vitamin B_6 metabolism have been established for decades, but little is known about how the concentration of PLP is controlled in mammalian tissues. The factors contributing to the regulation of PLP may include the following: catabolism of PLP by activities of PLP phosphatase, pyridoxal kinase (PK) and pyridoxine-5'-phosphate oxidase (PO), the degree of protein binding of the synthesized coenzyme and transport of the precursors (3, 4). Although PO plays a kinetic role in regulating the level of PLP formation (5, 6), PLP availability is mainly dependant on protein binding and phosphatase action (3, 7, 8).

A PLP-specific phosphatase catalyzes the dephosphorylation of PLP as well as PNP, and has been purified from human erythrocytes (9). Also, alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2) hydrolyze PLP and pyridoxamine 5'-phosphate (PMP), but they have broad substrate specificity for phosphomonoesters (10-12). We have previously reported that a human and mouse brain PLP phosphatase (PLPP) was cloned and characterized (13). The cDNA sequence of the mouse PLPP showed a high degree of similarity with the human PLPP (95%). PLPP is of particular interest because of the intimate relationship of vitamin B_6 metabolism to neurological disorders. Several lines of evidence have indicated that convulsive seizures occur during instances of vitamin B_6 deficiency (14-16). Despite the essential roles of PLPP in the various vitamin B_6-related disorders, further investigations are necessary to examine structural and regulatory roles of PLPP.

Many researchers have demonstrated the successful delivery of full-length fusion proteins by protein transduction technologies (17-20). Several small regions of protein, called protein transduction domains (PTD), have been developed to allow the delivery of exogenous proteins into living cells. By using this protein transduction technology, we have reported how various fusion proteins were efficiently transduced into mammalian cells and animal tissues. These transduced fusion proteins markedly protected cells and tissues (21-24).

In this paper, we describe the transduction of full length PEP-1-PLPP fusion protein into PC12 cells by their biological activity. Our results suggest the possibility that the transduction of the PEP-1-PLPP fusion protein would be useful as a therapeutic agent for the various disorders related to this enzyme and vitamin B_6.

RESULTS AND DISCUSSION

Construction and purification of PEP-1-PLPP fusion proteins

To develop an expression system to achieve overexpression and allow the straightforward purification of PLPP, we constructed a PEP-1-PLPP expression vector containing consecutive cDNA se-

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quences encoding human PLPP, PEP-1 peptide and six histidine residues at the amino-terminus (Fig. 1A). We also constructed the PLPP expression vector to produce a control PLPP protein without PEP-1 transduction peptides (data not shown).

Following the induction of expression, the PLPP fusion proteins were purified. Next, 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 the *E. coli* and purified PLPP fusion protein was electrophoresed in 12% SDS-PAGE. As shown in Fig. 1B and 1C, the PLPP fusion protein was highly expressed and found to be nearly homogenous and greater than 95% pure, as determined by SDS-PAGE. The purified products were further confirmed by Western blot analysis using a rabbit anti-histidine polyclonal antibody (dilution 1:500).

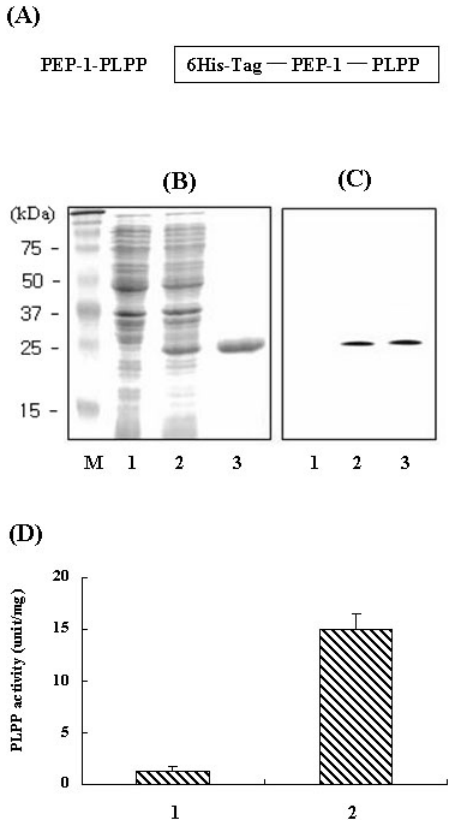

Fig. 1. Purification of the PEP-1-PLPP fusion protein. A schematic representation of the PEP-1-PLPP fusion protein (A). Expressed and purified PEP-1-PLPP fusion protein was analyzed by 12% SDS-PAGE (B) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (C). Lanes B and C are as follows: lane 1, non-induced; lane 2, induced; lane 3, purified. Enzymatic activity of the purified PEP-1-PLPP fusion protein (D). Lanes D are as follows: lane 1, crude extract; lane 2, purified.

The activity of the PEP-1-PLPP fusion protein was measured according to the method described by Jang *et al*. (13). As shown in Fig. 1D, the crude extract containing 0.13 units/mg of PLPP activity, and the purified PEP-1-PLPP fusion protein, had a specific activity of 1.5 units/mg. Moreover, Jang *et al*. (13) demonstrated that the purified recombinant PLPP protein had a specific activity of 1.4 units/mg of PLPP activity. Our results indicate that the purified PEP-1-PLPP fusion protein have an identical enzymatic activity level.

Transduction of PEP-1-PLPP fusion protein into PC12 cells

To evaluate the transduction ability of the PEP-1-PLPP fusion proteins, they were added to a culture media containing PC12 cells at various times and concentrations, and analyzed by Western blotting. As shown in Fig. 2A, the PEP-1-PLPP fusion protein was transduced into PC12 cells at a dose-dependent manner. Next, the PEP-1-PLPP fusion protein was added to PC12 cells in culture at various concentrations (0.5-5.0 μM) for 1 h, followed by analyzing the levels of transduction by Western blotting. The time-dependency for the transduction of the PEP-1-PLPP fusion protein was further analyzed. The PEP-1-PLPP (3 μM) fusion protein was added to the PC12 cell culture at various times (5-60 min), and the levels of transduction were analyzed by Western blotting. As shown in Fig. 2B, the intracellular concentration of the transduced PEP-1-PLPP

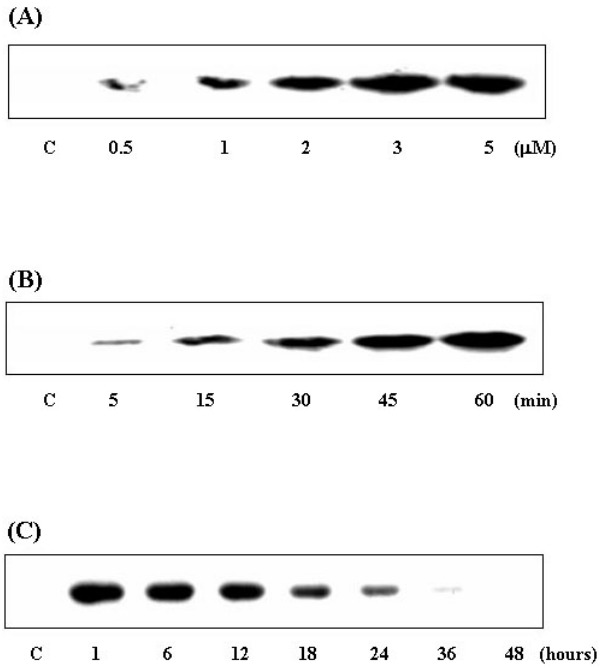

Fig. 2. Transduction of the PEP-1-PLPP fusion protein into PC12 cells. PEP-1-PLPP (0.5 - 5.0 μM) were added to the culture media for 1 h (A), 3 μM PEP-1-PLPP were added to the culture media for 5 - 60 min (B), and the cells pre-treated with 3 μM PEP-1-PLPP incubated for 1 - 48 h, and analyzed by Western blot analysis (C).

gradually increased. In addition, the intracellular stabilities of the transduced PEP-1-PLPP fusion protein in the PC12 cells are shown in Fig. 2C. We determined that significant levels of transduced PEP-1-PLPP fusion protein persisted in the PC12 cells for 36 h. Morris *et al*. (18) showed that PEP-1 peptide/GFP (green fluorescent protein, 30 kDa) or β-Gal (β-galactosidase, 119 kDa) mixtures transduce into a human fibroblast cell line (HS-68) and into Cos-7 cells by incubation with the PEP-1 peptide carrier and proteins (GFP or β-gal) for 30 min at 37°C. These differences in the time courses of transduction may depend on whether the target protein is fused with the PEP-1 vector or mixed with the PEP-1 peptide. Because of the fusion with the PEP-1 vector, the conformation, polarity and molecular shape of a target protein may be altered, which would improve the transduction of fusion proteins into cells.

The intracellular delivery of PEP-1-PLPP into the PC12 cells was confirmed by direct fluorescence. To exclude the possibility that cell fixation with paraformaldehyde may affect PEP-1-PLPP transduction by direct fluorescence, we transduced the FITC-labeled PEP-1-PLPP protein into non-fixed PC12 cells. As shown in Fig. 3, immunofluorescence staining using a rabbit anti-histidine polyclonal antibody, revealed that the PEP-1-PLPP fusion protein was transduced into the cells, whereas the fluorescence signals were absent in cells not treated with the PEP-1-PLPP fusion protein. In addition, the fluorescence signals of fixed cells were similar to those of non-fixed cells (data not shown). The results indicate that cell fixation is not required for PEP-1-PLPP fusion protein transduction.

The HIV-1 Tat and $(Arg)_9$ PTD fusion proteins are not transduced across the cell membrane of living cells, and any apparent entry is an artifact caused by fixation. Furthermore, fixation disrupts the cell membrane and therefore cannot be reliably used to study membrane-translocating proteins, since peptides and fusion proteins are internalized by endocytosis. Thus, fixation should be avoided in studies of protein transduction into living cells (25). However, in this study, we were unable to detect any differences in the fluorescence distribution of the transduced PEP-1-PLPP in non-fixed and fixed cells. Similar observations indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation (17, 26). We have also observed that the transduction of the PEP-1-SOD and Tat-SOD fusion proteins into neuronal and insulin-producing cells were not affected by paraformaldehyde fixation (22, 24).

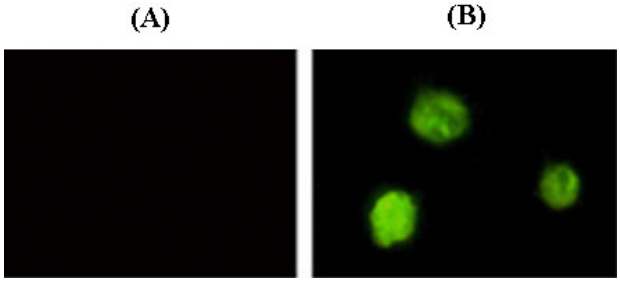

Fig. 3. Transduction of PEP-1-PLPP fusion protein into PC12 cells. Next, the FITC-labeled PEP-1-PLPP fusion proteins (3 μM) were transduced into PC12 cells and immediately observed by fluorescence microscopy. Control cells (A) and cells treated with PEP-1-PLPP (B).

Changes of PLP concentration by transduced PEP-1-PLPP fusion protein

To determine whether the transduced PEP-1-PLPP played its biological role in the cells, we tested the activity of transduced PEP-1-PLPP by adding pyridoxine (vitamin B_6), pyridoxal kinase (PK) and pyridoxine-5′-phosphate oxidase (PO) to PC12 cells. After pre-treating the cells with Tat-PK (2 μM) and Tat-PO (2 μM), the fusion proteins were exposed to 2 mM of pyridoxine, and the formation of PLP was significantly increased by about 9-fold. However, the concentration of PLP was markedly decreased when PEP-1-PLPP was added to the PC12 cells in culture media (Fig. 4). This result suggests that the PEP-1-PLPP fusion protein dephosphorylated the cofactor, in the form of PLP, into pyridoxal. Moreover, the concentration of pyridoxal increased as a function of transduced PEP-1-PLPP concentration.

Pyridoxal-5′-phosphase (PLP), known as a biologically active form of vitamin B_6, serves as a cofactor required by numerous enzymes which are involved in the catalysis of transamination and decarboxylation reactions (2, 27). The formation of PLP depends upon the catalytic functions of two enzymes, pyridoxal kinase (PK) and PNP oxidase (PO). In a previous study, we showed that the transduced Tat-PK and Tat-PO increased the intracellular concentration of PLP. Moreover, PLP formation increased synergistically when Tat-PK and Tat-PO were co-transduced, than by transduction of either, alone (28, 29).

In summary, the present study demonstrated that exogenous human PLPP fused with PEP-1 peptides, which can be directly transduced into PC12 cells, and that the resulting enzymatically active PLPP converts PLP into pyridoxal. Thus, we can conclude that the transduction of the PEP-1-PLPP fusion protein may offer a therapeutic tool for the various disorders re-

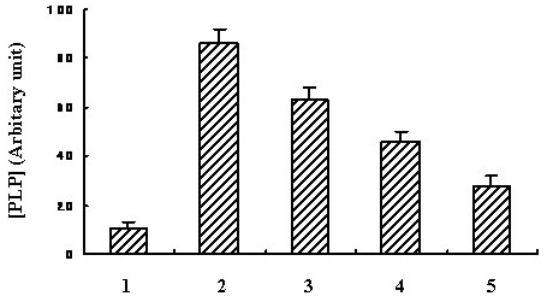

Fig. 4. Changes in PLP concentration by the transduced PEP-1-PLPP in PC12 cells pretreated with the vitamin B_6 precursor pyridoxine, PK and PO. The bars are as follows: lane 1, PC12 cell only; lane 2, PK + PO + 2 mM pyridoxine; lane 3, PK + PO + 2 mM pyridoxine + PLPP (1 μM); lane 4, PK + PO + 2 mM pyridoxine + PLPP (2 μM); lane 5, PK + PO + 2 mM pyridoxine + PLPP (3 μM).

lated to vitamin B_6 metabolism.

MATERIALS AND METHODS

Materials

Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen (GmbH, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. (Haarlen, Netherlands). Fetal bovine serum (FBS), RPMI1640 and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). Pyridoxine, ATP and goat anti rabbit immunoglobulins were purchased from Sigma (St. Louis, MO, USA). The rabbit anti-histidine polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were purchased at the highest analytical grade available.

Construction and purification of PEP-1-PLPP fusion protein

The construction of the PEP-1 fusion protein plasmid has been previously described (24). Briefly, the pyridoxine-5′-phosphate phosphatase (PLPP) gene was amplified by PCR with the two following primers: The sense primer was 5'-CTCGAGGCGCG CTGCG-3' containing an *Xho*I restriction site, and the antisense primer was, 5'-GGATCCTCAGTCCTCCA ACCCC-3' containing a *Bam*HI restriction site. Next, the PCR products were purified and cloned into a TA cloning vector. After digesting with *Xho*I and *Bam*HI, the PEP-1-PLPP gene was ligated into the expression vector, and pET-15b in frame with a six histidine open-reading frame to generate the expression vector. The PEP-1-PLPP in the pET-15b vector was expressed in *E. coli* BL21 (DE3). Next, the host *E. coli* BL21 (DE3) was transformed with plasmids encoding the PEP-1-PLPP, followed by the selection of transformants 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 cell growth to an $O.D._{600}$ = 0.5 - 0.6, the protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM with ongoing incubation for an additional 3 - 4 h. The cells were harvested and 5 ml of a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) was added, followed by sonication. Following a centrifugation step, the supernatants containing PEP-1-PLPP were immediately loaded onto a 2.0 ml Ni^{2+}-nitrilotriacetic acid Sepharose column. After the column was washed 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 with an elution buffer (0.5 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. Lastly, the protein concentrations were estimated by the Bradford procedure using bovine serum albumin as a standard (30).

Transduction of PEP-1-PLPP into cultured PC12 cells

The 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 under a humidified condition of 95% air and 5% CO_2. The transduction of PEP-1-PLPP consisted of growing PC12 cells to confluence on a 6-well plate. Next, the culture media was replaced with 1 ml of fresh solution. The PC12 cells were then treated with various concentrations of PEP-1-PLPP for 1 h. The cells were then treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). Finally, the cells were harvested and the cell extracts were prepared for PEP-1-PLPP Western blot analysis.

Enzymatic assay of PLPP

The enzyme assay of PLPP was performed according to the method described by Jang *et al*. (13). The enzymatic activity of PLPP was measured at pH 7.4 in 40 mM triethanol-amine-HCl. Moreover, the rate of pyridoxal production from PLP was measured by following the decrease in absorbance at 390 nm for at least 3 min. One unit of specific activity was defined as the amount of protein that catalyzes the formation of pyridoxal/min from PLP.

Fluorescence microscopy

For the direct detection of fluorescein-labeled protein, purified PEP-1-PLPP was labeled using an EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (PIERCE, Rockford, USA). The FITC labeling was carried out according to the manufacturer's instructions. The PC12 cells were grown on glass coverslips and treated with 3 μM of PEP-1-PLPP fusion protein for 1 h at 37°C, and then washed with trypsin-EDTA treated PBS. The fluorescence distributions were analyzed using a Zeiss Axiophot fluorescence microscope.

Determination of PLP concentration

The cells were pre-treated with a 2 mM vitamin B_6 precursor pyridoxine for 3 h and washed three times with PBS to remove free pyridoxine. The 2 μM Tat-pyridoxal kinase (PK), 2 μM Tat-pyridoxine-5′-phosphate oxidase (PO) and PEP-1-PLPP (1-3 μM) were transduced into the cells for 1 h. The concentrations of free PLP in the cells were measured by the spectroscopic method described previously (29, 31)

Western blot analysis

For the Western blotting, proteins in the cell extracts were separated on a 12% SDS-PAGE, followed by the transferring of the proteins onto a nitrocellulose membrane. The membranes were incubated for 1 h in block solution [5% nonfat dry milk in Tris-buffered saline (TBS)], and washed three times with TBS containing Tween 20. After washing, the membranes were incubated with rabbit anti-histidine polyclonal antibody (dilution 1:500) and anti-pyridoxine-5-P oxidase monoclonal antibody for 1 h at room temperature, washed three times with TBS containing Tween 20 and incubated with goat anti-rabbit im-

munoglobulins (Sigma, dilution 1:10,000) for 1 h. Lastly, the immunoreactive bands were visualized using an chemiluminescence (ECL) detection system (32).

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