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HIV-1 Tat-mediated protein transduction of human brain creatine kinase into PC12 cells

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Epilepsy is characterized by the presence of spontaneous episodes of abnormal neuronal discharges and its pathogenic mechanisms remain poorly understood. Recently, we found that the expression of creatine kinase (CK) was markedly decreased in an epilepsy animal model using proteomic analysis. A human CK gene was fused with a HIV-1 Tat peptide to generate an in-frame Tat-CK fusion protein. The purified Tat-CK 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 Tat-CK fusion protein was stable for 48 h. Moreover, the Tat-CK fusion protein markedly increased endogenous CK activity levels within the cells. These results suggest that Tat-CK provides a strategy for the therapeutic delivery of proteins in various human diseases including the delivery of CK for potential epilepsy treatment. [BMB reports 2008; 41(7): 537-541]

INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2), a member of the highly conserved phosphagen kinase enzyme family, catalyzes the reversible transfer of the γ-phosphoryl group of MgATP to creatine, resulting in the formation of phosphocreatine (PCr) and MgADP. CK plays a key role in energy metabolism in cells with high and variable ATPase activity levels such as neurons, muscle fibers, transport epithelia, and spermatozoa, and it is widely distributed in both invertebrate and vertebrate organisms (1-3). To date, four different isozymes of CK have been identified in mammalian tissues. There are two cytosolic CK subunits, brain (B-CK) and muscle (M-CK), which associate to form the brain and the muscle isozymes, BB-CK and MM-CK, respectively. There are also two forms of the enzyme (sarcomeric Mi-CK and ubiquitous Mi-CK) located in the inner membrane of mitochondria. The human muscle, brain, and mitochondrial CK cDNAs all show a high degree of homology, with each encoding a protein of about 40 kDa (3, 4-9).

It is well known that CK is an important clinical marker for a number of neuronal disease states. Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that alteration of CK activity may represent an important step in neurodegenerative pathways that lead to neuronal loss in the brain (10-12). Several studies have shown that CK activity is significantly reduced in some neurodegenerative diseases. In addition, oxidative modification and impaired CK function may contribute to the loss of CK activity in neurodegenerative diseases (13-15).

Many researchers have demonstrated the successful delivery of full-length fusion proteins by protein transduction technology (16-19). Several small regions of proteins, 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 previously reported on the efficient transduction of various fusion proteins into mammalian cells and animal tissues. The transduced fusion proteins markedly protected cells and tissues against reactive oxygen species (20-23).

Epilepsy is a chronic condition characterized by the presence of spontaneous episodes of abnormal neuronal discharges. An imbalance between excitatory and inhibitory transmission is considered to be the most common cause of epilepsy (24-26). Recently, we found that the expression and enzymatic activity of CK was markedly decreased in an epilepsy animal model using proteomic analysis.

In this paper, we describe the transduction of a Tat-CK fusion protein into PC12 cells and the resulting biological activity of the protein. Our results suggest the possibility that transduction of this Tat-CK fusion protein would be useful as a therapeutic agent for various disorders related to CK function including epilepsy.

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RESULTS AND DISCUSSION

Construction and purification of Tat-CK fusion proteins

To develop an expression system for the overexpression and straightforward purification of CK, we constructed a Tat-CK expression vector containing consecutive cDNA sequences encoding the human brain CK subunit, the HIV-1 Tat peptide and six histidine residues at the amino-terminus (Fig. 1). We also constructed a CK expression vector in order to produce control CK protein without the HIV-1 Tat transduction peptide (data not shown).

The Tat-CK fusion protein was expressed in *E. coli* and was purified from cell extracts following expression induction. The clarified extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid sepharose affinity column. Fractions containing the fusion protein were combined and salts were removed using a PD10 column. The crude cell extracts and purified CK fusion proteins were analyzed by 12% SDS-PAGE. As shown in Fig. 2, the CK fusion protein was highly expressed and was purified nearly to homogeneity, yielding a band that was 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).

The purified Tat-CK fusion protein had a specific activity of 146.2 units/mg. Towler *et al*. (27) demonstrated that purified recombinant CK exhibited a specific activity of 140.6 unit/mg. In addition, the specific activity of the recombinant CK protein was 50% higher than that purified from human brain tissue. Our results indicate that the purified Tat-CK fusion protein has identical enzymatic activity to purified recombinant CK.

Transduction of the Tat-CK fusion protein into PC12 cells

The intracellular delivery of FITC-labeled Tat-CK into PC12 cells was confirmed by direct fluorescence. To exclude the possibility that cell fixation with paraformaldehyde may affect Tat-CK transduction assayed by direct fluorescence, we transduced FITC-labeled Tat-CK protein into non-fixed PC12 cells. As shown in Fig. 3, immunofluorescence using a rabbit anti-histidine polyclonal antibody revealed that the Tat-CK fusion protein was transduced into the cells, whereas fluorescence signals were absent in cells not treated with the Tat-CK fusion

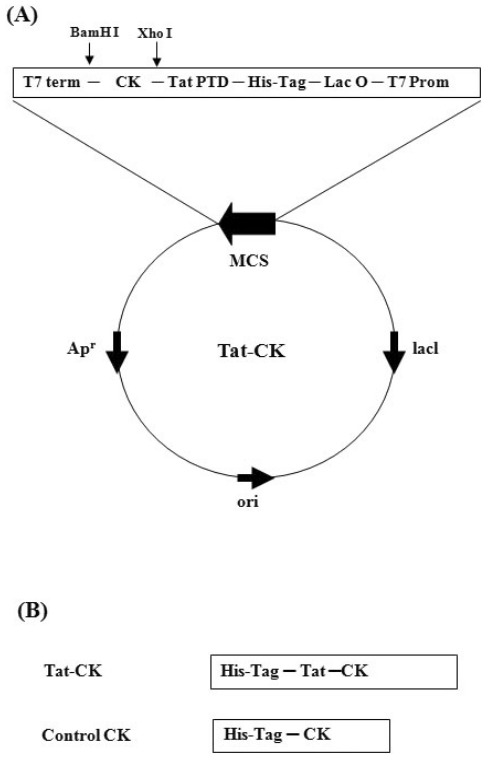

Fig. 1. Construction of a Tat-CK expression vector based on the vector, pET-15b. A synthetic HIV-1 Tat oligomer was cloned into the *Nde*I and *Xho*I sites and a human CK cDNA was cloned into the *Xho*I and *Bam*HI sites of pET-15b (A). Diagram of the expressed Tat-CK fusion protein. The coding frame of CK is represented by an open box along with the 6His tag and the HIV-1 Tat peptide (B). Expression was induced by adding IPTG.

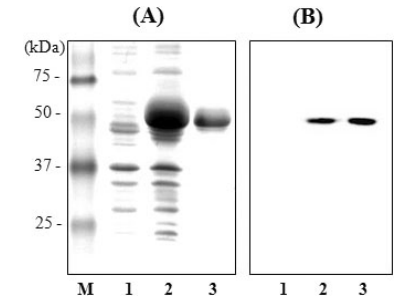

Fig. 2. Purification of the Tat-CK fusion protein. Expression and purification of the Tat-CK fusion protein were examined by 12% SDS-PAGE (A) and by western blot analysis with an anti-rabbit polyhistidine antibody (B). Lanes in A and B are as follows: lane 1, non-induced; lane 2, induced; lane 3, purified.

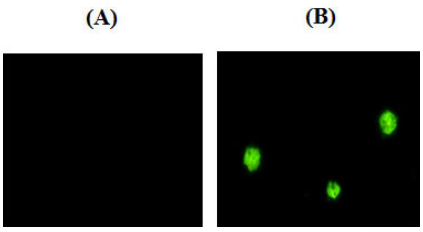

Fig. 3. Detection of the transduced Tat-CK fusion protein in PC12 cells. After the FITC-labeled Tat-CK fusion protein (3 μM) was transduced into PC12 cells, the cells were washed with trypsin-EDTA followed by PBS and were immediately observed by fluorescence microscopy. Control cells (A) and cells treated with Tat-CK (B).

protein. In addition, fluorescence signals detected in fixed cells were similar to those in non-fixed cells (data not shown). These results indicate that cell fixation is not required for Tat-CK fusion protein transduction.

HIV-1 Tat and an $(Arg)_9$ PTD fusion protein are not transduced across the cell membrane of living cells, and any apparent entry is an artifact caused by fixation. 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 (28). However, in this study, we were unable to detect any differences in the fluorescence distribution of transduced Tat-CK in non-fixed and fixed cells. Similar observations indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation (29). We have also observed that the transduction of PEP-1-SOD and Tat-SOD fusion proteins into neuronal and insulin-producing cells, respectively, was not affected by paraformaldehyde fixation (21, 23).

To evaluate the transduction efficiency of the Tat-CK fusion protein, different concentrations (1-3 μM) of purified Tat-CK fusion protein were added to the culture media of PC12 cells for various times and the cells were analyzed by western blotting. As shown in Fig. 4A, the Tat-CK fusion protein was transduced into PC12 cells in a dose-dependent manner following a incubation. The time-dependence of Tat-CK transduction was further analyzed. The Tat-CK (3 μM) fusion protein was added to PC12 cells in culture for various incubation times (15-60 min), and the levels of transduction were analyzed by western blotting. As shown in Fig. 4B, the intracellular concentration of Tat-CK gradually increased with increasing incubation periods. In addition, we examined the stability of the transduced Tat-CK fusion protein in PC12 cells by western blotting. PC12 cells were incubated with 3 μM Tat-CK for 1 h, extracellular Tat-CK was removed by washing, and the cells were incubated for an additional 60 h. Intracellular Tat-CK levels declined gradually over the period of observation but significant levels of the transduced Tat-CK fusion protein persisted in PC12 cells for 48 h (data not shown). It was reported that a Tat-β-galactosidase fusion protein was rapidly transduced into HepG2 cells, reaching nearly maximal intracellular concentrations in less than 15 min (18). The small difference in this time course as compared to ours may result from the properties of the transduced Tat fusion protein, such as the degree of folding, the polarity, and the molecular shape of the protein.

We determined the activity of CK in PC12 cells transduced with the Tat-CK fusion protein. As shown in the lower panels of Fig. 4A and 4B, CK activity levels increased in a time- and dose-dependent manner in the Tat-CK-transduced PC12 cells. Intracellular CK activity levels increased six-fold after treatment with 3 μM Tat-CK for 60 min. However, treatment of PC12 cells with the CK control did not lead to an increase in intracellular CK activity levels (data not shown). These results demonstrate that Tat-CK is efficiently transduced into PC12 cells and enhances their CK activity levels. Choi *et al*. (5) demonstrated that CK activity is decreased by reactive oxygen species (ROS). The inactivation of CK by ROS may be involved in the regulation of energy metabolism in cancer cells. In addition, Aksenov *et al*. (13) demonstrated that CK expression levels and enzymatic activity levels were lower in brain tissue samples from Alzheimer's disease (AD) patients than in control brain tissue samples. Oxidative modification of CK and impaired CK function may contribute to the loss of CK activity in AD.

In summary, the present study demonstrated that exogenous human brain CK fused with HIV-1 Tat peptide, can be directly transduced into PC12 cells, and that the resulting intracellular CK activity levels are significantly increased. Thus, we conclude that transduction of the Tat-CK fusion protein may offer a therapeutic tool for various disorders related to CK function including epilepsy and AD.

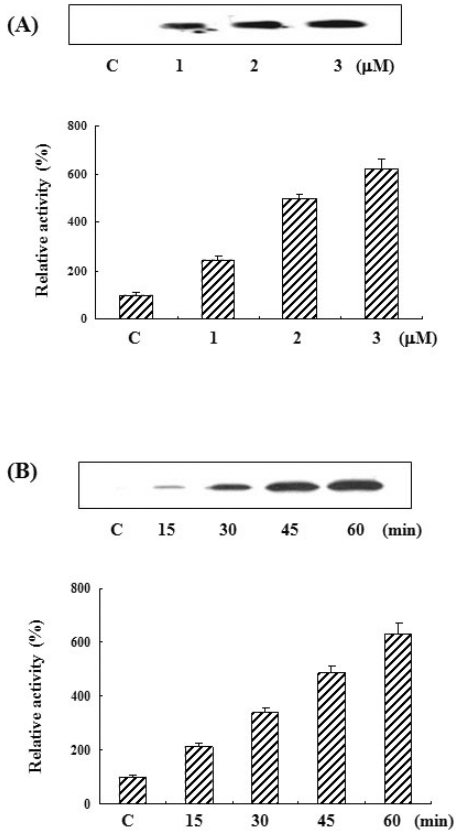

Fig. 4. Concentration- and time-depedence of Tat-CK transduction into PC12 cells. Tat-CK (1-3 μM) was added to the PC12 culture media for 1 h (A). Tat-CK (3 μM) was added to the PC12 culture media for 15-60 min (B). The levels of transduced Tat-CK fusion protein were analyzed by western blotting (upper panel) and enzyme activity assays (lower panel).

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, NY, USA). The rabbit anti-histidine polyclonal antibody was purchased from the Santa Cruz Biotechnology company (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest analytical grade available.

Construction and purification of Tat-CK fusion protein

Construction of the HIV-1 Tat fusion protein plasmid was previously described (20). The human brain creatine kinase (CK) gene was amplified by PCR with two primers. The sense primer, 5'-CTCGAGATGCCCTTCAACAGC-3', contained an *Xho*I restriction site, and the antisense primer, 5'-GGATCCTCATTT CTGGGCAGGC-3', contained a *Bam*HI restriction site. The resulting PCR product was purified and cloned into a TA cloning vector. After digesting with *Xho*I and *Bam*HI, the Tat-CK gene was ligated into the expression vector, pET-15b in frame with a six-histidine tag to generate the expression vector.

The Tat-CK fusion protien was expressed in *E. coli* BL21 (DE3). The host *E. coli* BL21 (DE3) was transformed with the expression plasmid encoding the Tat-CK fusion protein and transformants were selected on a LB plate containing ampicillin. The selected colonies were cultured in LB medium containing ampicillin at 37°C with shaking at 250 rpm. Following cell growth to an $O.D_{600}$ = 0.5-0.6, protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM and the incubation was continued for 3-4 h. The cells were harvested and 5 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea were added. The cell suspension was then sonicated and centrifuged. After centrifugation, supernatants containing Tat-CK were immediately loaded on 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 wash 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. The protein concentrations were estimated by the Bradford procedure using bovine serum albumin as a standard (30).

Transduction of Tat-CK 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 under a humidified atmosphere of 95% air and 5% CO_2. For the transduction of Tat-CK, PC12 cells were grown to confluence on a 6-well plate. Then, the culture media were replaced with 1 ml of fresh solution. PC12 cells were subsequently treated with various concentrations of Tat-CK for 1 h. The cells were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). They were then harvested and cell extracts were prepared for Tat-CK western blot analysis.

Enzymatic assay of CK

CK enzyme activity was measured using a CK assay kit according to the manufacturer's instructions (5, 31).

Fluorescence microscopy

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

Western blot analysis

For western blotting, cell extracts were separated by 12% SDS-PAGE and the proteins were transferred to 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 (1:500 dilution) for 1 h at room temperature and washed three times with TBS containing Tween 20. The membranes were then incubated with goat anti-rabbit immunoglobulins (Sigma, dilution 1:10,000) for 1 h. Immunoreactive bands were visualized using a chemiluminescence (ECL) detection system (32).

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