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Generation and Characterization of Cell-Permeable Green Fluorescent Protein Mediated by the Basic Domain of Human Immunodeficiency Virus Type 1 Tat

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Abstract The human immunodeficiency virus type 1 (HIV-1) Tat is one of the viral gene products essential for HIV replication. The exogenous Tat protein is transduced through the plasma membrane and then accumulated in a cell. The basic domain of the Tat protein, which is rich in arginine and lysine residues and called the protein transduction domain (PTD), has been identified to be responsible for this transduction activity. To better understand the nature of the transduction mediated by this highly basic domain of HIV-1 Tat, the Green Fluorescent Protein (GFP) was expressed and purified as a fusion protein with a peptide derived from the HIV-1 Tat basic domain in *Escherichia coli*. The transduction of Tat-GFP into mammalian cells was then determined by a Western blot analysis and fluorescence microscopy. The cells treated with Tat-GFP exhibited dose- and time-dependent increases in their intracellular level of the protein. The effective transduction of denatured Tat-GFP into both the nucleus and the cytoplasm of mammalian cells was also demonstrated, thereby indicating that the unfolding of the transduced protein is required for efficient transduction. Accordingly, the availability of recombinant Tat-GFP can facilitate the simple and specific identification of the protein transduction mediated by the HIV-1 Tat basic domain in living cells either by fluorescence microscopy or by a fluorescence-activated cell sorter analysis.

Key words: Expression, HIV-1, Tat, GFP, transduction

As a transcription transactivator produced by HIV-1 during the early phase of infection, the Tat protein plays a critical role in the expression and replication of the viral genome [1, 15, 22]. The HIV-1 Tat protein contains at least three functional domains: an acidic group of amino acids at the amino terminus, a cysteine-rich cluster consisting of seven cysteine residues, and a positively-charged region rich in arginine and lysine residues [18].

The Tat protein, which is secreted from infected cells, has an ability to enter neighboring cells through the plasma membrane and then accumulates in these cells [5, 8, 9, 13]. Due to this property, the whole Tat protein and parts of it have been tested for their ability to deliver several proteins, including ovalbumin, beta-galactosidase, and horseradish peroxidase, into cells [6, 27], and it has been determined that the basic domain of the Tat protein, which is rich in arginine and lysine residues, is responsible for this property [25]. This domain is also required for both the nuclear and the nucleolar localization of the Tat protein [10, 19]. The short basic domain of Tat, called the protein transduction domain (PTD), has been recently shown to serve as a carrier that directs the uptake of heterologous proteins into cells by generating genetic in-frame PTD fusion proteins [16, 26]. Futhermore, it has also been reported that, when injected intraperitoneally into mice, beta-galactosidase fused to the basic domain of HIV-1 Tat was delivered in a biologically active form to all tissues including the brain [21]. The function of HIV-1 PTD in the process termed protein transduction signifies the usefulness of the HIV-1 whole Tat protein or the short basic domain of Tat responsible for transduction activity as a delivery vehicle of biologically active molecules that are non-permeable into cells. Although it has been suggested that transduction occurs in a receptor- and transporter-independent fashion that appears to target the lipid bilayer directly, the mechanism of the transduction mediated by the HIV-1 PTD needs to be elucidated [4, 25]. When this technique become generally available, several important questions on the fraction of cells affected, the treatment period, the degree of uptake into cells, reproducibility, and the location of the proteins within the cells can be addressed. Subsequently, this domain could then be utilized as a reliable general technique to efficiently deliver a variety of

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biologically functional target proteins into large numbers of living cells or organisms for the treatment of genetic disorders, cancer, and neurological diseases, as well as the study of cell biology and drug delivery.

In this report, to better understand the nature of the transduction mediated by the basic domain of HIV-1 Tat (amino acids 49-57), the green fluorescent protein (GFP) was chosen as a model protein. An expression vector (pTat-GFP) was constructed containing GFP cDNA genetically fused on its N-terminus with a fragment encoding the basic domain of HIV-1 Tat. Tat-GFP fusion proteins were prepared in native and denatured forms and then the cellular uptake and their subcellular localization of these proteins were investigated. The transduction efficiency of Tat-GFP in denatured form was higher than that of the native form. The transduction of Tat-GFP into cells was dose- and time-dependent, as determined by a Western blot analysis. Fluorescence microscopy of fixed cells showed green fluorescent signals in nearly 100% of the cells treated with Tat-GFP.

MATERIALS AND METHODS

Construction of Expression Vectors

pTat-GFP was constructed to express the basic domain (amino acids 49-57) of HIV-1 Tat as a fusion with GFP as follows: First, two oligonucleotides were synthesized and annealed to generate a double-stranded oligonucleotide encoding 9 amino acids from the basic domain of HIV-1 Tat. The sequences were 5'-TAGGAAGAAGCGGAGAC-AGCGACGAAGAC-3' (top strand) and 5'-TCGAGTCT-TCGTCGCTGTCTCCGCTTCTTCC-3' (bottom strand). The double-stranded oligonucleotide was directly ligated into *Nde*I-*Xho*I digested pET15b (Invitrogen, Carlsbad, U.S.A.) in frame with a 6His open reading frame to generate the HisTat expression plasmid, pHisTat. The sequences of the polylinkers cloned into the plasmid were confirmed with a fluorescence-based automated sequencer (model 373A; Applied Biosystems, Inc.). Next, the complete *gfp* sequence was amplified from plasmid pEGFP-C2 (Clontech) using a polymerase chain reaction (PCR) [17]. The PCR was performed using *Pfu* DNA polymerase (Clontech) [23]. The sense primer was: 5'-CTCGAGGTGAGCAAG-GGCGAGGAGCTG-3' and antisense primer: 5'-GGATC-CTTACTTGTACAGCTCGTCCATGCCGAG-3'. The PCR product was digested with *Xho*I-*Bam*HI and subcloned into the *Xho*I-*Bam*HI sites of the pHisTat vector. Similarly, the PCR product digested with *Xho*I-*Bam*HI was subcloned into the *Xho*I-*Bam*HI sites of pET15b to construct pGFP that expressed the GFP fusion protein without the basic domain of HIV-1 Tat. The clones with the expected 0.7 kb insert were selected using an *Xho*I-*Bam*HI restriction analysis and then analyzed by sequencing [20].

Expression and Purification of Tat-GFP Fusion Proteins

BL21 *E. coli* (Pharmacia) transformed with plasmids encoding GFP or Tat-GFP fusion proteins were grown overnight at 37°C in an LB broth supplemented with 100 μg/ml ampicillin. The overnight culture was diluted ten-fold with fresh LB media and cultured at 37°C, while shaking at 250 rpm until OD_{600}=1.0. The protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM for 4 h. To prepare the denatured Tat-GFP fusion proteins, the induced cells were harvested and lysed by sonication in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea and protease inhibitors (20 mg/ml soybean trypsin inhibitor, 2 mg/ml aprotinin, 5 mg/ml leupeptin, and 100 mg/ml PMSF). After the removal of the cell debris by centrifugation, the clarified cell extract was then loaded onto an Ni^{++}-IDA column [12]. The column was washed first with the binding buffer without 6 M urea and then with a wash buffer (80 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The proteins were eluted by the buffer (1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9), followed by desalting with a PD10 column (Amersham). The native Tat-GFP fusion protein was obtained by the same procedure as described above without the denaturing agent. The protein concentrations in each fraction were quantitated by a densitometric analysis after separation by SDS-PAGE using bovine serum albumin (BSA) as the standard. The protein concentrations were determined with a Bradford protein assay (Biorad) using BSA as the standard [3]. The purified GFP fusion proteins dissolved in PBS containing 20% glycerol were then aliquoted and stored at - 80°C.

Cell Culture and Transduction of Tat-GFP

The HeLa cells were cultured in a Dulbecco's Modified Eagle's Medium containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS), and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C. For the transduction of Tat-GFP, the HeLa cells were grown to confluence on a 6-well plate. Thereafter, the culture medium was replaced with a fresh DMEM containing 10% FBS, which was then treated with various concentrations of Tat-GFP. After incubating at 37°C for 1 h, the cells were washed with phosphate buffered saline (PBS) and trypsinized for 10 min (Gibco BRL). The cells were harvested for the preparation of cell extracts to perform a Western blot analysis.

Western Blot Analysis

Cell lysates were prepared by lysing monolayer cells on a six-well plate with lysis buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol). For the Western blot analysis, 15 μg of proteins from each whole cell lysate were loaded onto a 12% SDS-polyacrylamide gel. The proteins were

electrotransferred to a nitrocellulose membrane, which was then blocked with 10% dry milk in phosphate-buffered saline (PBS). The membrane was probed with a rabbit anti-GFP polyclonal antibody (Clontech, dilution 1:1,000), followed by incubation with goat anti-rabbit immunoglobulins (Sigma, dilution 1:10,000). The bound antibodies were then visualized by an enhanced chemiluminescence according to the manufacturer's instruction (ECL; Amersham).

Analysis of Transduced Cells by Fluorescence Microscopy

HeLa cells grown on coverslips to 50 to 70% confluency were treated with varying amounts of Tat-GFP. Following incubation for 1 h, the cells were washed twice with PBS, trypsinized, and fixed in 3.7% formaldehyde in PBS for 5 min at room temperature. The cells were washed again with PBS before being mounted in PBS containing 90% glycerol and 0.1% phenylediamine. The distribution of the fluorescence was analyzed using an Olympus epifluorescence microscope with a 488 nm fluorescent filter [11].

RESULTS

Construction of Tat-GFP Fusion Protein Expression Vector

Previous studies implicated that the basic domain of HIV-1 Tat is involved in the transduction activity of Tat. This basic domain consisted of six arginine amino acids, two lysines, and a glutamine. The green fluorescent protein (GFP) was used as a model protein fused with the basic domain of HIV-1 Tat to confirm that it can mediate the transduction of foreign proteins into cells. The green fluorescent protein from the jellyfish *Aequorea victoria* is one of the more widely used reporter proteins in the analysis of protein targeting or trafficking in cells [24].

To construct an expression vector to express the Tat-GFP fusion protein, two oligonucleotides were initially designed to generate a sequence to encode the 9-amino acid residues of the positively-charged basic domain of HIV-1 Tat. The resulting vector was named pHisTat. Next, the coding sequence for GFP was amplified using the PCR method and inserted to downstream of the basic sequence of HIV-1 Tat into a pHisTat vector using the *Xho*I and *Bam*HI sites. The resultant pTat-GFP vector was thus encoded for a *gfp* as a fusion protein with the basic domain of HIV-1 Tat (Fig. 1A). To generate a control GFP fusion protein without the basic sequence of HIV-1 Tat, a GFP expression vector, pGFP, was also constructed by inserting the coding sequence for GFP into pET15b. The Tat-GFP and GFP fusion proteins were both designed with an N-terminal histidine hexapeptide, thereby allowing for the simple purification and concentration of the recombinant proteins from the bacterial lysates by affinity chromatography using a metal chelating matrix (Fig. 1B).

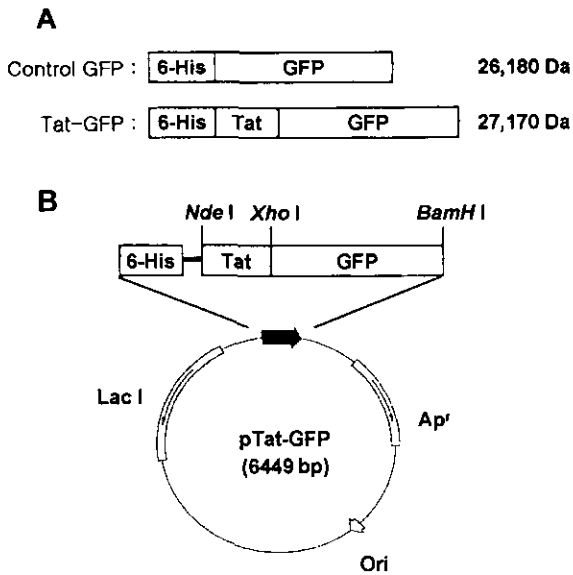

Fig. 1. Tat-GFP fusion protein and expression vector.
(A) Diagram of Tat-GFP and control GFP fusion proteins. The coding frame of GFP is represented by an open box along with 6His and the HIV-1 Tat basic domain (RKKRRQRRR). (B) Tat-GFP expression vector. The PCR-amplified cDNA fragments containing the GFP coding sequences were cloned in frame into pHisTat containing the coding sequence of the HIV-1 Tat basic domain. The resulting vectors were designated pTat-GFP. Ap', ampicilin resistance gene; Ori, plasmid replication origin; Lac I, lac repressor expressing gene.

Expression and Purification of Recombinant Tat-GFP Fusion Proteins

The concentration of IPTG and induction period were varied to find out the optimal conditions for the expression

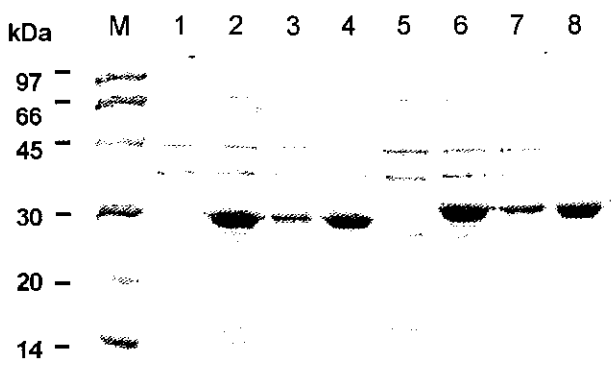

Fig. 2. Determination of solubility of GFP fusion proteins.
E. coli BL21 transformed with pGFP or pTat-GFP was induced with 0.5 mM IPTG for 3 h. Cell pellets from the induced bacterial culture were lysed directly in 50 mM Tris-HCl, pH 8.0 containing lysozyme (100 μg/ml) and fractionated into soluble and insoluble fractions. The bacterial proteins in each fraction were analyzed by 12% polyacrylamide-SDS gel electrophoresis and detected by Coomassie Brilliant blue staining. Lane M, Molecular weight markers; lanes 1 and 5, pre-induction culture of *E. coli* BL21 transformed with pGFP or pTat-GFP, respectively; lanes 2 and 6, expression culture *E. coli* BL21 transformed with pGFP or pTat-GFP, respectively, 3 h after induction with IPTG; lanes 3 and 7, supernatant; lanes 4 and 8, pellet.

of the recombinant HIV-1 Tat proteins. It was examined whether the fusion proteins expressed by the pTat-GFP or pGFP vectors in *E. coli* were synthesized as soluble or insoluble proteins. *E. coli* transformed with each vector was induced with IPTG and lysed by sonication. The soluble and insoluble cell fractions from the cell lysates were prepared and analyzed by gel electrophoresis with Coomassie Brilliant blue staining. The Tat-GFP and GFP fusion proteins were both found to be present mainly as an insoluble fraction following the lysis of the bacterial cells (Fig. 2).

Following the induction of expression, the cell lysates containing either Tat-GFP or GFP were prepared under denaturing conditions or native conditions. The GFP fusion proteins containing six histidine residues at the N terminus were affinity purified by metal chelate chromatography. The purification results of the chromatographic fractions are shown in Fig. 3A. An analysis of the purified Tat-GFP protein by SDS-PAGE showed that Tat-GFP migrated at a higher apparent molecular mass than the GFP protein which lacked the Tat sequence, due to the presence of the Tat basic domain which has a calculated molecular mass of 990 Da. The protein was found to be nearly homogeneous and >80% pure, as determined by an SDS-PAGE analysis with Coomassie Brilliant blue staining (Fig. 3B). The purified products were further confirmed by a Western blot analysis using a rabbit polyclonal antibody to GFP (Fig. 3B). The Tat-GFP and GFP fusion proteins were detected at the corresponding bands, respectively. The yields of the fusion proteins were approximately 2 mg/l culture routinely. These preparations of Tat-GFP proteins were then used for further biological experiments.

Comparison of Translocation Efficiency of Native and Denatured Tat-GFP Proteins

In the present study, the Tat-GFP fusion proteins purified under denaturing conditions or native conditions were used to compare their translocation activity, respectively, along with the control GFP. The native or denatured Tat-GFP proteins were added to culture media of HeLa cells for 1 h at a concentration of 0.5 μM and the presence of transduced proteins was analyzed by Western blotting

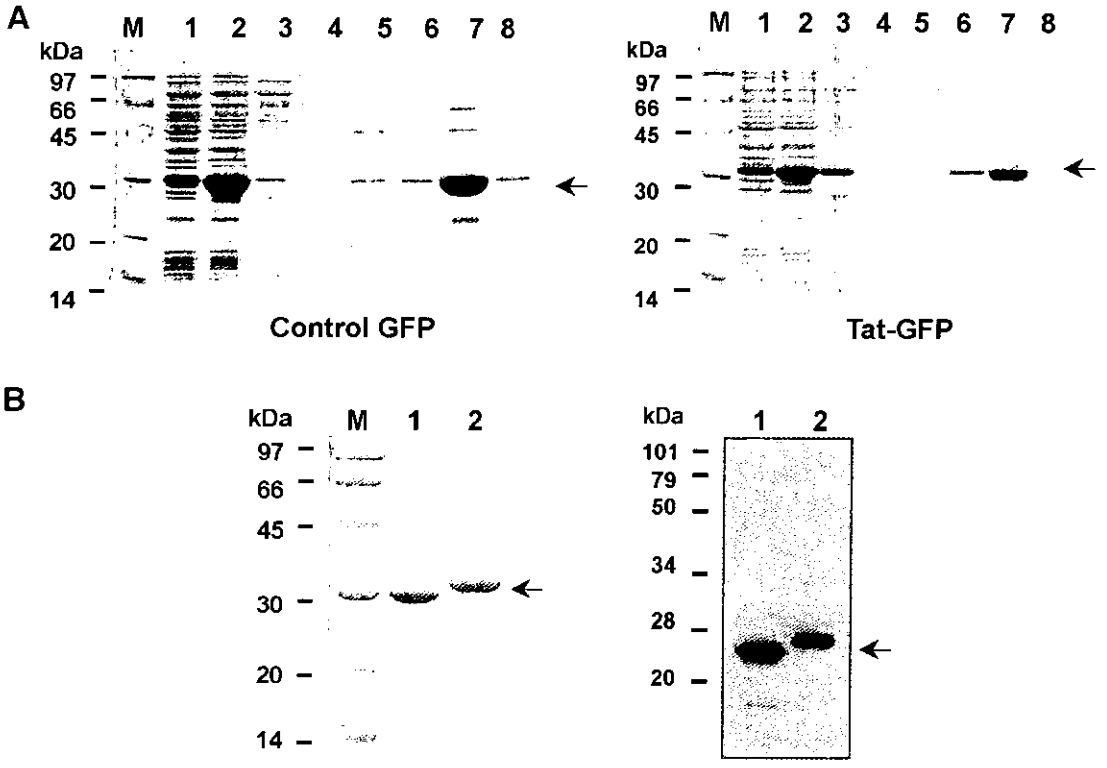

Fig. 3. Analysis of GFP fusion proteins during purification steps.
(A) *E. coli* BL21 transformed with pGFP or pTat-GFP was induced with 0.5 mM IPTG for 3 h and fractionated to purify the recombinant fusion protein by affinity chromatography on a nickel sepharose column. Aliquots of each fraction were removed from several steps of the purification and analyzed by 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant blue. In each panel, lane 1, pre-induction culture of transformed *E. coli* BL21; lane 2, expression culture after induction; lane 3, flow-through fraction from NTA column; lane 4, fraction washed with binding buffer; lane 5, fraction washed with washing buffer; lanes 6-8, proteins eluted with elution buffer containing 1 M imidazole. (B) Coomassie Brilliant blue staining and Western blot analysis of purified GFP fusion proteins. The purified fusion proteins were separated by a 12% SDS-PAGE gel and either stained with Coomassie Brilliant blue or then transferred to nitrocellulose membranes. The membranes were incubated with a rabbit polyclonal antibody to GFP as the first antibody and then goat-anti rabbit IgG as the secondary antibody. The blot was developed by the ECL method. Lane 1, control GFP; lane 2, Tat-GFP.

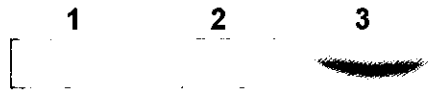

Fig. 4. Comparison of transduction efficiency of native and denatured Tat-GFP proteins.
HeLa cells plated in a 6-well plate were treated with either native Tat-GFP or denatured Tat-GFP at a concentration of 0.5 μM. One-hour after treatment, the cells were harvested and washed. The transduction activity of each protein was measured by analyzing the level of the transduced protein in the cells by a Western blot analysis. Lane 1, control GFP; lane 2, native Tat-GFP; lane 3, denatured Tat-GFP.

using a rabbit polyclonal antibody to GFP. As shown in Fig. 4, the native and denatured Tat-GFP were readily detected in the cells, whereas no control GFP was detected in the cells, thereby indicating the the GFP protein alone is not capable of transducing into the cells. The level of transduced protein in the cells treated with the Tat-GFP fusion proteins in native form became significantly lower than that of the denatured form. This result indicates that the preparation of the Tat-GFP fusion proteins in native form resulted in a dramatic reduction in their transduction activity.

Transduction Profiles of Denatured Tat-GFP Fusion Protein

The kinetics of the transduction of the denatured Tat-GFP fusion proteins was analyzed. The denatured Tat-GFP proteins were added to culture media of HeLa cells at a concentration of 0.5 μM for various time periods and the resulting levels of transduced proteins were analyzed by Western blotting. As shown in Fig. 5A, the intracellular level of transduced Tat-GFP into the cultured cells was initially detected after 5 min, peaked after 30 min, and then remained the same for 6 h. The amounts of transduced Tat-GFP in the cells were estimated to be several hundreds

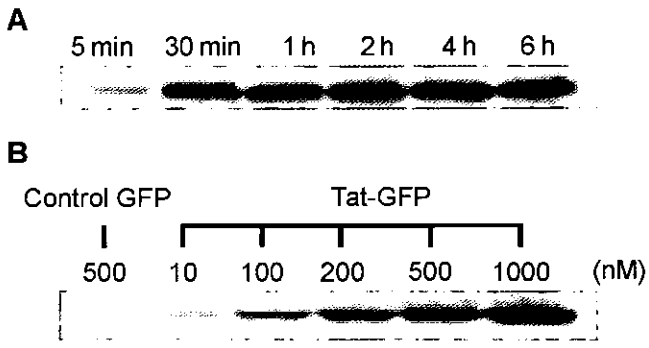

Fig. 5. Analysis of transduced protein by denatured Tat-GFP protein.
(A) Time-dependent analysis of transduced protein. The denatured Tat-GFP proteins were added at a concentration of 0.5 μM to HeLa cells for 5 min, 30 min, 1 h, 2 h, 4 h, and 6 h. At different time points after treatment, the cells were harvested. The presence of the transduced protein was analyzed by a Western blot analysis. (B) Dose-dependent analysis of transduced protein. Varying concentrations of Tat-GFP were added to HeLa cells for 1 h and the amounts of proteins transduced into the cells were measured.

nanograms of protein per 5×10^5 cells, as judged from the band intensities of the Western blot analysis using purified Tat-GFP as the standard. Thereafter, to analyze the dose dependency of the transduction of the denatured Tat-GFP fusion proteins, the denatured Tat-GFP proteins were added to culture media of HeLa cells at various concentrations for 1 h and the resulting levels of the transduced proteins were analyzed by Western blotting. As shown in Fig. 5B, the level of the transduced proteins in the cells treated with denatured Tat-GFP increased concomitantly with the amount of protein treated into the cells. In contrast, the GFP lacking the Tat basic domain was not transduced into the cells under the same experimental conditions. The time- and dose-dependent manner of transduction indicates that Tat-GFP was rapidly and efficiently transduced into the cells.

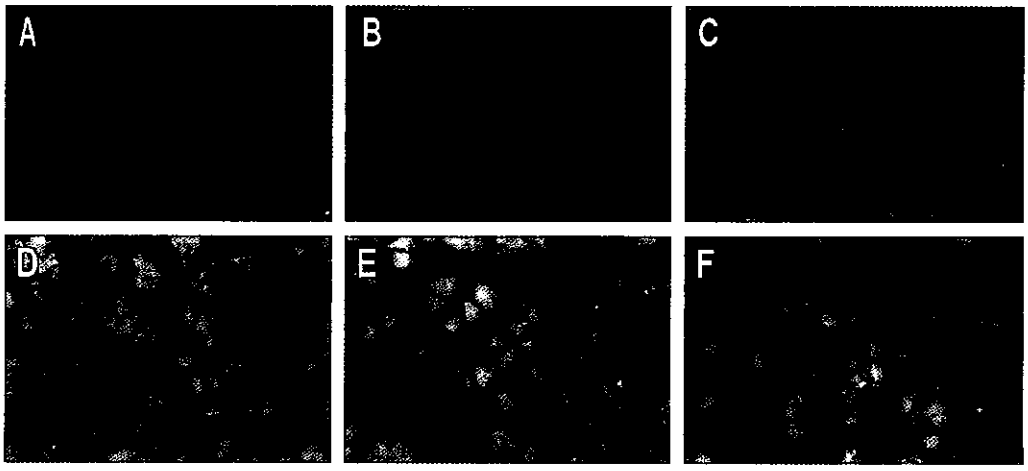

Fig. 6. Visualization of Tat-GFP transduced cells by fluorescence microscopy.
Microphotography section (original magnification, × 200) of HeLa cell treated with various concentrations of Tat-GFP protein for 1 h. (A) 500 nM control GFP; (B) 10 nM Tat-GFP; (C) 100 nM Tat-GFP; (D) 200 nM Tat-GFP; (E) 500 nM Tat-GFP; (F) 1000 nM Tat-GFP.

Biological Activity of Transduced Tat-GFP Fusion Protein

To investigate whether the denatured Tat-GFP transduced into the cells is correctly refolded and restored its biological activity, the fluorescence of the transduced Tat-GFP into the cells was analyzed using fluorescence microscopy. As shown in Fig. 6, the level of the fluorescence signal in the cultured HeLa cells treated with denatured Tat-GFP increased concomitantly with the amount of protein treated into the cells. A linear correlation was observed between the level of the fluorescent signal and the amount of protein treated. As can be seen in Fig. 6, when the cells were treated with low concentrations of Tat-GFP, the fluorescence was distributed to the cytosol and nucleus. When the cells were treated with high concentrations of Tat-GFP, the Tat-GFP was mainly redistributed in the nucleus and partly in the cytoplasm within 1 h. When GFP lacking the Tat basic domain was treated into the cells at a high concentration, the fluorescence signal was low compared to the background level. Taken together, these data confirm that the transduced denatured Tat-GFP was refolded into a biologically active conformation in the mammalian cells.

DISCUSSION

Previous studies have shown that HIV-1 Tat can be released and freely entered cells, yet still retained its activity, thereby enabling it to up-regulate a number of genes [5, 27]. The basic domain of Tat is responsible for not only translocation but also nuclear localization [19]. As such, the basic domain of HIV-1 Tat, called the protein transduction domain (PTD), has great potential to be used as a vehicle that can deliver non-permeable compounds into cells. This possibility has already been tested using ovalbumin, beta-galactosidase, horseradish peroxidase, and caspase-3 [6, 16, 26]. Accordingly, with the use of the Tat-GFP fusion proteins, several questions can be asked for the transduction mediated by the HIV-1 PTD; the nature of the transduced protein, the fraction of the cells affected, the treatment period, the extent of the uptake between cells, the reproducibility, and the location of the proteins within the cells. In these experiments, GFP fused to the basic domain of HIV-1 Tat (amino acids 49-57) expressed in *E. coli* was tested for its cellular uptake and subcellular localization in the cells, when added extracellularly. It was demonstrated that the exogenously denatured Tat-GFP expressed in *E. coli* could be directly transduced into mammalian cells across a lipid membrane barrier in a time- and dose-dependent manner, independently and without any other factor.

The purpose of this study was to set up a purification protocol that can be readily used to obtain partially denatured fusion proteins with efficient transduction activity, as demonstrated in this study and previous experiments [16]. To produce partially denatured fusion proteins, cells were first disrupted in 6 M urea, then the cell lysates were immediately subjected to purification in the absence of urea. This procedure, as described in this study, enables the production of partially denatured, soluble proteins competent for transduction into cells. Although the degree of the denaturing status of the purified Tat-GFP fusion protein has not yet been investigated, a significant increase in the translocation activity of the fusion protein purified under denaturing conditions was observed compared with that of the fusion protein purified under native conditions. This result indicates that the purified Tat-GFP fusion protein retains at least a partially denatured form and suggests that it is necessary to remove the denaturants quickly, as implicated in previous studies, thereby showing that denatured proteins would appear to transduce more efficiently into cells than the correctly folded proteins in terms of the structural constraint involved in crossing cellular membranes. Therefore, this step would appear more appropriate when it is essential to obtain fusion proteins competent with efficient transduction activity.

The observation that the denatured protein had a better transduction efficiency than the native protein was consistent with previous results using other denatured Tat fusion proteins [16, 21]. It has been suggested that the increased transduction efficiency of denatured Tat-GFP may result from reduced structural constraints which then allow the proteins to pass through the cell membrane more easily [16]. The evidence supporting this comes from a previous study that demonstrated that when Tat is used to ferry dihydrofolate reductase into cells, unfolding is necessary to transverse the cellular membrane [2]. Future studies are needed to further address this property.

The time course of the transduction of the Tat-GFP fusion protein given in Fig. 5 showed that the maximum intracellular level of the transduced protein was achieved at about 30 min after the incubation, indicating that Tat-GFP was rapidly transduced into the cells. In contrast to this observation, the Tat-beta-galactosidase fusion protein has been shown to transduce rapidly into HepG2 cells, reaching near maximum intracellular concentrations in less than 15 min [21]. This slight discrepancy in the transduction kinetics may derive from the properties of the transduced Tat fusion protein, including the unfolding degree, polarity, and molecular shape of the protein.

The restoration of the biological activity of the protein transduced into the cells is a prerequisite for the application of protein transduction technology to a biological system. The result shown in Fig. 6 indicates that the denatured Tat-GFP in HeLa cells can be correctly refolded in mammalian cells by spontaneous processes or by molecular chaperones such as heat shock proteins [7, 14]. However, the refolding mechanisms of transduced exogenous proteins in cells remains to be elucidated. The Tat-GFP protein was localized

in both the cytoplasm and the nucleus after being transduced into cells, as observed by fluorescence microscopy. It has been previously suggested that this cluster of 9 basic amino acids (Tat 49-57) serves as a nuclear localization signal [19]. From the results analyzing the dose dependency of the transduction of Tat-GFP fusion proteins by fluorescence microscopy, a difference in the cellular localization of the transduced protein was observed depending on the amount of protein extracellularly added. In cells treated with low concentrations of Tat-GFP, the fluorescence signal was observed as being mainly distributed in the cytosol and only partly in the nucleus, whereas in cells treated with high concentrations of Tat-GFP, the Tat-GFP was redistributed mainly in the nucleus and only partly in the cytoplasm within 1 h. Therefore, a preferential localization of transduced protein was obtained by altering the concentration of the proteins. In addition, the transduction of Tat-GFP into the cells was highly efficient, as confirmed by observing the fluorescent signals of the cells treated with Tat-GFP, where almost 100% of the cells were positive.

Other Tat fusion proteins such as beta-galactosidase or luciferase could be developed to examine the cellular distribution of transduced proteins within cells [21]. However, the requirement of exogenous substrates or cofactors to assay the biological activity often limits the availability of these proteins. As shown in this study, the Tat-GFP fusion protein was easily used to localize transduced proteins in cells with fluorescence microscopy without staining or the requirement of exogenous compounds. Consequently, the availability of Tat-GFP facilitates the analysis of the transduction activity mediated by the basic domain of HIV-1 Tat as well as the cellular localization of the transduced protein. In addition, Tat-GFP can be used in a variety of applications including studies of *in vivo* and *in vitro* protein delivery systems. Further studies using Tat-GFP molecules are necessary for elucidating the mechanism of the transduction mediated by the basic domain of the HIV-1 Tat protein. Taking advantage of the ability of Tat-GFP to be detected by fluorescence microscopy in cells, efforts are currently being made to delineate the optimal sequence required for the transduction of target proteins into cells.

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