The Journal of Microbiology, December 2004, p.328-335 Vol. 42, No. 4

Characteristics of HIV-Tat Protein Transduction Domain

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(Received September 15, 2004 / Accepted October 5, 2004)

The human immunodeficiency virus type 1 (HIV-1) Tat protein transduction domain (PTD), which contains rich arginine and lysine residues, is responsible for the highly efficient transduction of protein through the plasma membrane. In addition, it can be secreted from infected cells and has the ability to enter neighboring cells. When the PTD of Tat is fused to proteins and exogenously added to cells, the fusion protein can cross plasma membranes. Recent reports indicate that the endogenously expressed Tat fusion protein can demonstrate biodistribution of several proteins. However, intercellular transport and protein transduction have not been observed in some studies. Therefore, this study examined the intercellular transport and protein transduction of the Tat protein. The results showed no evidence of intercellular transport (biodistribution) in a cell culture. Instead, the Tat fusion peptides were found to have a significant effect on the transduction and intercellular localization properties. This suggests that the HIV-1 PTD passes through the plasma membrane in one direction.

Key words: HIV-1-Tat, protein trasduction domain, biodistribution

Gene therapy using viral vectors, such as a baculovirus and adenovirus, is an attractive tool for treating cancer. However, the main problem with tumor gene therapy is the low rate of tumor cell transudation *in vivo* using the vectors available.

Recently, several small regions of proteins, called protein transduction domains (PTDs), have been identified as carriers for the efficient delivery of proteins that do not permeate living cells. Although the mechanism is unknown, transduction occurs in receptor- and transporter-independent manners, which appears to target the lipid bilayer directly (Steven *et al.*, 2000). PTDs include the peptides derived from the basic domain of HIV-1 Tat, the homeodomain of *Drosophila Antennapedia* (Antp) and the HSV VP22 transcription factor (Joliot *et al.*, 1991; Derossi *et al.*, 1994; Elliott and O'Hare *et al.*, 1997; Han *et al.*, 2000; Wender *et al.*, 2000; Futaki *et al.*, 2001; Park *et al.*, 2002).

The well-characterized HIV-1 PTD of Tat has potentially enhanced utility relative to other PTDs owing to its smaller size of 11 aa. This short Tat peptide, YG**RKKRRQRRR** (residues 47-57), which is rich in basic amino acids (highlighted in bold), is sufficient for the intracellular transduction and subcellular localization (Vives *et al.*, 1997; Nagahara *et al.*, 1998). It can deliver a wide variety of proteins, ranging in size from 15 to 120 kDa, across the plasma membrane by a mechanism referred to as "protein transduction" (Frankel and Pabo *et al.*, 1988; Green and Loewenstein *et al.*, 1988; Vives *et al.*, 1997). It has been suggested that transduction by HIV-1 Tat does not occur in a classical receptor, transporter or endosome-mediated manner (Joliot *et al.*, 1991; Derossi *et al.*, 1994). An inherent characteristic of the specific sequences or numbers of the amino acids arginine or lysine, or some structural motif, may be important in this process (Vives *et al.*, 1997; Schwarze and Dowdy *et al.*, 2000; Ho *et al.*, 2001). Significantly, Tat maintains its nascent properties of post-intercellular trafficking. This observation led to the hypothesis that the HIV-1 Tat might be a useful molecular tool for achieving the widespread distribution of recombinant proteins in gene therapy applications (Schwarze and Dowdy *et al.*, 2000). Several groups have previously demonstrated the endogenous expression of a Tat fusion peptide *in vivo,* followed by intercellular trafficking. In these studies, Tat was fused to β-glucuronidase and expressed from a recombinant adenoviral vector, and when the Tat was fused to β-galactosidase, a significantly enhanced "biodistribution" of β-galactosidase was observed, both *in vitro* and *in vivo*. However, a recent report has shown apparently contradictory results (Xia *et al.*, 2001; Elliger *et al.*, 2002). These studies have found no evidence of the intercellular transport of Tat fusion

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peptides. If Tat has biodistribution ability, the Tat fusion peptides may overcome a limitation of the viral mediate gene delivery. By increasing the distribution through intercellular transport, more cells can potentially reach the levels of the therapeutic proteins necessary for efficacy, leading to an overall enhancement of the biological effects in the target population (Bao *et al.*, 1996; Gao *et al.*, 1996; Lieber *et al.*, 1997).

This study examined the transduction efficiencies of the exogenously expressed Tat fusion EGFP (bacteria system), and the biodistribution ability of the endogenously expressed Tat fusion EGFP (baculovirus system).

Materials and Methods

Cell culture

The insect cell line, Sf9, was grown in Grace's medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, USA). The human hepatoma cell line, Huh7, the human cervical carcinoma line, HeLa, and the human glioblastoma, A172, were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, USA), supplemented with 10% FBS and 4 mM glutamine. The cultures were maintained at 37°C in a humidified atmosphere of 95% air/ 5% CO_2.

Construction of plasmids expressing EGFP fusion proteins

In order to construct the plasmid with the HIV-1 Tat fused to the C-terminus of EGFP (pEGFP-Tat), two oligonucleotides were synthesized and annealed to generate a double stranded oligo -nucleotide encoding the 11 amino acids from the basic domain of the HIV-1 Tat. The sequences were (top strand) 5' GATCT**AGAAGCAGC GACAGAGGCGAAGAAGGACGGTATTAAC**T 3' and (bottom strand) 5' A**TCTTCGTCGCTGTCTCCG CTTCTTCCTGCCATAATT**GACAGCT 3'. The double stranded oligonucleotide was inserted into pCR *2.1* (Invitrogen, USA) in order to generate pCR 2.1-Tat. Second, the EGFP gene sequence was amplified using PCR from pEGFP-N1 (Invitrogen, USA), with the sense primer 5' AGAATCCGC-TAGCGCTACCGGTCGCCACCCATGG 3' and antisense primer 5' GAAGATCTCTTGTACAGCTCGTCCAT 3'. The *Eco*RV/*Bgl*II EGFP fragment of the PCR product and the *Eco*RV /*Bgl*II Tat fragment of pCR 2.1-Tat were subcloned into the *Nde*I/*Bam*HI sites of pET-15b (Clontech, USA), generating pEGFP-Tat (Fig. 1A). The ABI Prism automated sequencing method was used to confirm the nucleotide sequences of all the PCR products.

Expression and purification of EGFP fusion proteins

The *E. coli* BL21 cells transformed with the plasmids encoding the EGFP fusion proteins were grown to an OD_{600} of 1 at 37°C in LB medium containing ampicillin (100 μg/ml). Protein expression was induced by the addition of IPTG, to a final concentration of 0.5 mM, for 4 h. In order to prepare the denatured EGFP fusion proteins, the induced cells were harvested and lysed by sonication in a pH 7.9 binding buffer (5 mM imidazole, 500 mM

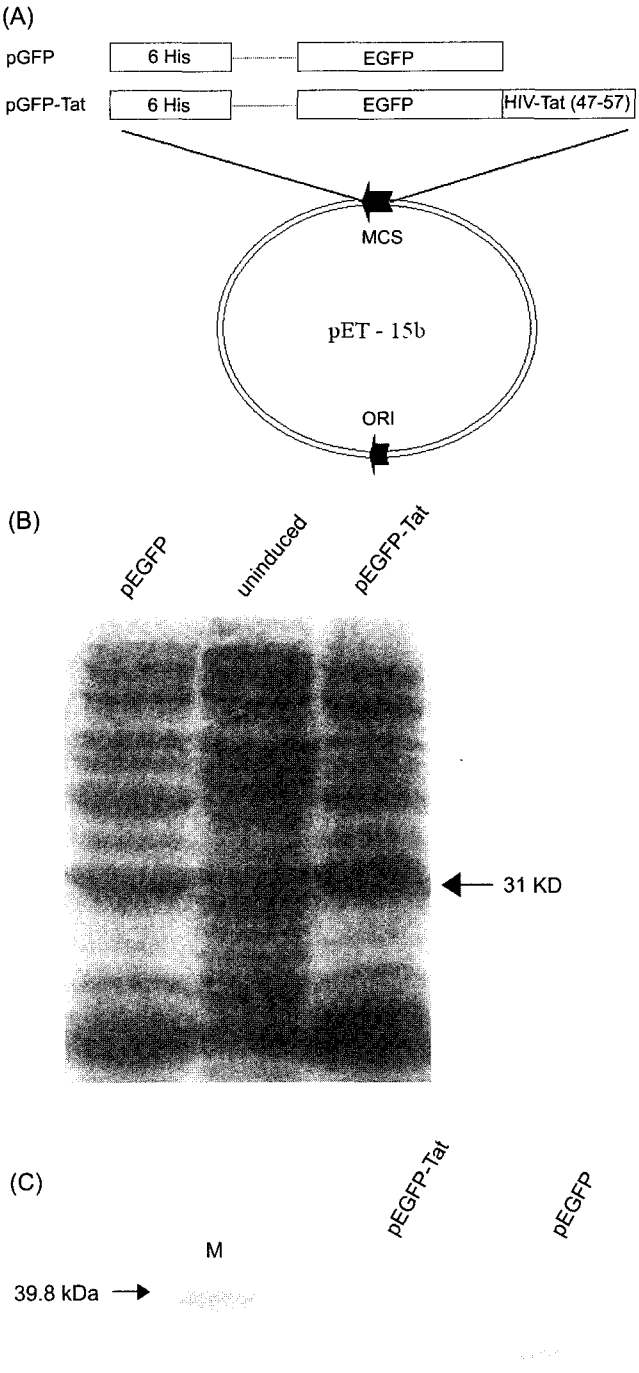

Fig. 1. (A) Schematic map of the EGFP fusion protein expression vectors. The expression vectors, pEGFP and pEGFP-Tat, were constructed as described in **Materials and Methods**. (B) Expression of EGFP, with or without Tat, in *E. coli*; lane 1, induced EGFP; lane 2, uninduced control; lane 3, induced EGFP-Tat. (C) Analysis of the purified EGFP fusion proteins. Fusion proteins purified using a Ni^{2+} affinity chromatography column were separated by 10% SDS-PAGE and stained with Coomassie blue. Lane M, molecular mass markers; lane 1, pEGFP-Tat; lane 2, pEGFP.

NaCl and 20 mM Tris-HCl) containing 6 M urea. The proteins were purified using Ni^{2+} affinity chromatography (Park *et al.*, 2002). The purified EGFP and Tat fusion EGFPs were equally fluorescent (data not shown), indicating that the Tat EGFP fusion proteins expressed in *E. coli* had the correct conformation and were active (Park *et al.*, 2002; Silhol *et al.*, 2002). The protein concentrations were determined using a Bradford protein assay kit (Bio-rad, USA), with bovine serum albumin as the standard (Bradford, 1976). Aliquots of the purified EGFP fusion proteins were added to PBS containing 20% glycerol and stored at 80°C (Ryu *et al*,. 2003).

Transduction of the fusion protein into cells

When the cells were 70% confluent, the culture medium was replaced with fresh medium containing 10% FBS, and the EGFP or EGFP-Tat added to the growth medium, to a final concentration of 0.5 μM. The cells were then sampled at the times shown or after at least 10 min.

Generation of recombinant baculoviruses

The baculovirus plasmid, Bac-EGFP-Tat, was generated by inserting the expression cassette into the standard baculovirus transfer vector, pBlueBac4.5 (Invitrogen, USA). First, the VSV-G gene was excised as a 1665-bp *Xho*I/*Eco*RI fragment from the full-length VSV gene cDNA. This fragment was inserted into the *Xho*I/*Eco*RI site of pBlueBac 4.5 in a direct orientation with respect to the polyhedrin promoter to create pBlueBacG. Subsequently, in order to generate pCEP4-EGFP-Tat, the Tat fuion EGFP gene was released by *Xba*I/*Xho*I double digestion from the pEGFP-Tat inserted into the *Sal*I/*Xho*I sites of pCEP4 (Invitrogen, USA). The integrity of the Tats-EGFP fusion was confirmed by sequencing. A 2.5-kb *Sal*I fragment (CMV promoter cassette) from pCEP4-EGFP-Tat was cloned into *Sna*BI-digested pBlueBacG, generating Bac-EGFP-Tat (Fig. 2A). The recombinant viruses were generated using a Bac-To-Bac Baculovirus Expression System (Gibco BRL, USA). The virus was generated also using the Bac-to-Bac system (Invitrogen, USA). The virus was further amplified by propagation in Sf9 (*Spodoptera frugiperda*) cells grown in suspension of Grace's supplemented insect media, containing 10% (vol/vol) fetal bovine serum, 0.1% (vol/vol) pluronic F-68 and 25 mg/ml gentamycin, according to standard protocols (O'Reilly *et al.*, 1992). Stocks of virus were concentrated by centrifugation at 35,000×*g* for 60 min, and the pelleted virus resuspended in Dulbecco's PBS, supplemented with 1% (vol/vol) fetal bovine serum. The virus titers were determined by a plaque assay on Sf9 cells.

Transduction of mammalian cells by Bac-EGFP-Tat, EGFP virus

The cells were seeded so that they were approximately 80-90% confluent at the time of infection. Infections were carried out in supplemented MEM medium (serum free media) in either 24-well tissue culture plates, or 6 or 9cm tissue culture plates, in total volumes of 200 μl, 500 μl or 1 ml, respectively. Infections were left for 1 h at 37°C, and supplemented MEM medium (10% fetal bovine serum) then added to total volumes of 0.5, 4 or 10 ml, respectively.

Western blotting analysis

The samples were prepared for Western blot analysis using the following method. The Sf9 cells were infected with Bac-EGFP, Bac-EGFP-Tat m.o.i. (multiplicity of infection) 10, in 6-well plates. After 48 h, the cells were lysed in a Laemmli buffer (125 mM Tris, 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue, pH 6.8) and heated to 100°C for 5 min prior to electrophoresis. All the samples were run on SDS-10% polyacrylamide gel electrophoresis. The VSV-G-specific monoclonal antibody was obtained from Roche Molecular Biochemicals. The presence of the VSV-G protein was detected using an ECL Western blotting analysis system (Amersham Bioscience, Sweden).

Fluorescence microscopy

The cultured cells were grown on glass coverslips, and then infected with the recombinant baculovirus (m.o.i. 10) for 1 h. Forty eight hour after infection, the cells were washed three times with 1 ml of PBS (pH 6.4), sealed in GelMount (Biomedia, USA), then viewed on an fluorescence microscope (Carl Zeiss, Germany) and recorded with an Axio camera (Carl Zeiss, Germany).

Flow cytometry

The cultures were harvested with trypsin 24 h after infection and transduction, then washed and resuspended in PBS supplemented with 1% fetal bovine serum. The data collection was performed by FACS Calibur flow cytometry (Becton Dickinson, USA).

Results

Construction and expression of Tat fusion EGFP vector

A peptide (RKKRRQRRR), derived from the HIV-1 Tat basic domain fused to the C-terminus of EGFP as recombinant fusion proteins, was produced to facilitate the visualization of the PTDs in cell cultures. A control EGFP expression vector was also constructed by inserting the coding sequence for EGFP into the pET15b expression vector (Fig. 1A). EGFP and EGFP-Tat were expressed or transformed *E. coli* BL21 (DE3) cells (Fig. 1B). These fusion proteins were found to be well expressed, even without IPTG induction. The fusion proteins could be easily monitored during the entire protein expression and purification process due to their green fluorescence. The

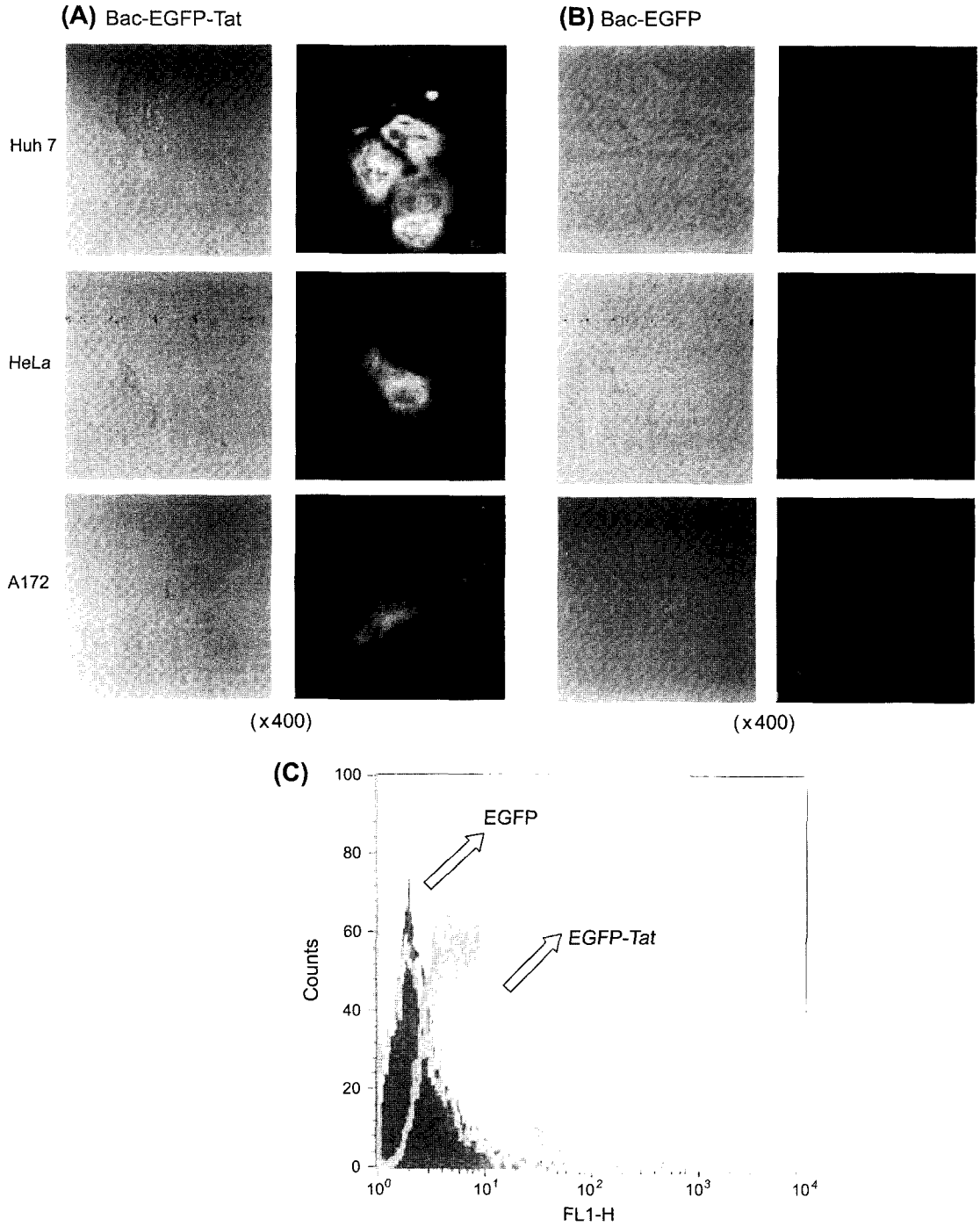

Fig. 2. Analysis of the transduction efficiencies of EGFP fusion proteins. (A) Subcellular localization of the transduced EGFP with, and (B) without, Tat proteins, as visualized by confocal microscopy. Cells were treated with 0.5 μM GFP fusion proteins for 15 min, and washed quickly with PBS. (C) Comparison of EGFP fusion proteins uptake in HeLa cells using FACS.

cell lysates containing the EGFP fusion proteins, with the His_6 tag at their N termini, were prepared under denaturing or native conditions. The fusion proteins were those purified by the Ni^{2+} affinity chromatography and determined by SDS-PAGE analysis with Coomassie Brilliant blue staining (Fig. 1C). The EGFP fusion proteins migrated at the expected sizes.

Transduction and intercellular localization efficiency of the Tat fusion EGFP proteins in the cells

The transduction efficiencies of the Tat fusion EGFP proteins, purified under denaturing conditions, were determined and compared with the control EGFP. As shown in Fig. 2A, EGFP-Tat was readily detected in the cells, whereas it was only weakly detected in the control EGFP.

The levels of the EGFP fusion protein in the cells were significantly higher in the EGFP-Tat fusion than the EGFP proteins. Flow cytometry of live cells is commonly used to examine HIV-1 Tat imports and to quantify the cellular import of these proteins. These results were also confirmed by flow cytometry (Fig. 2C). A high transduction efficiency of EGFP-Tat was also observed in the A172 and Huh7 cell lines (data not shown).

In order to determine if the HIV-1 Tat could affect the capability of intercellular localization, the transduced proteins in the cells were determined by confocal microscopy. EGFP-Tat was detected in the cytoplasm of the transduced cells, whereas the control EGFP was not detected in the cells (Fig. 2A).

Tat fusion EGFP expressed from baculovirus vector

DNA expression cassettes expressing EGFP-Tat regulated by the CMV promoter were constructed in order to determine the intercellular transport capability of the endogenously expressed Tat fusion proteins. These expression cassettes were inserted into the baculovirus vector (Fig. 3A). The recombinant baculovirus, pseudotyped with vesicular stomatitis virus (VSV) G protein, has been developed previously. The VSV-G gene, under the control of the polyhedrin promoter, was expressed at high levels in infected insect cells, but not in mammalian cells (Park *et al.*, 2001). The presence of the VSV-G protein in the purified baculovirus preparations was confirmed by Western blotting analysis (Fig. 3B). In order to confirm that the EGFP and Tat fusion EGFP protein were expressed from each virus, the Huh7 cells were infected with each of the recombinant baculoviruses, and the EGFP expression observed (data not shown).

Intercellular transport ability of endogenously expressed Tat fusion EGFP

Several cell lines were infected with comparable titers of the recombinant baculovirus in order to assess the intercellular transport capability of Tat fusion EGFP. It was considered better to evaluate the EGFP expression in living cells, which was accomplished, not only by fluorescence microscopy, but also by flow cytometry, which permits an objective and accurate quantitation of the number of cells expressing EGFP and the level of expression in each cell. As shown in Fig. 4A, the percentages of EGFP in all cell lines were quite similar. Moreover, similar results were also observed by flow cytometry (Fig. 4B). These results suggest that HIV-1 Tat does not lead to the transfer of the marker protein into non-infected cells. However, when the infected cells were fixed in methanol, a dramatic change was observed in the population of cells infected with the Tat fusion EGFP expressed virus, with

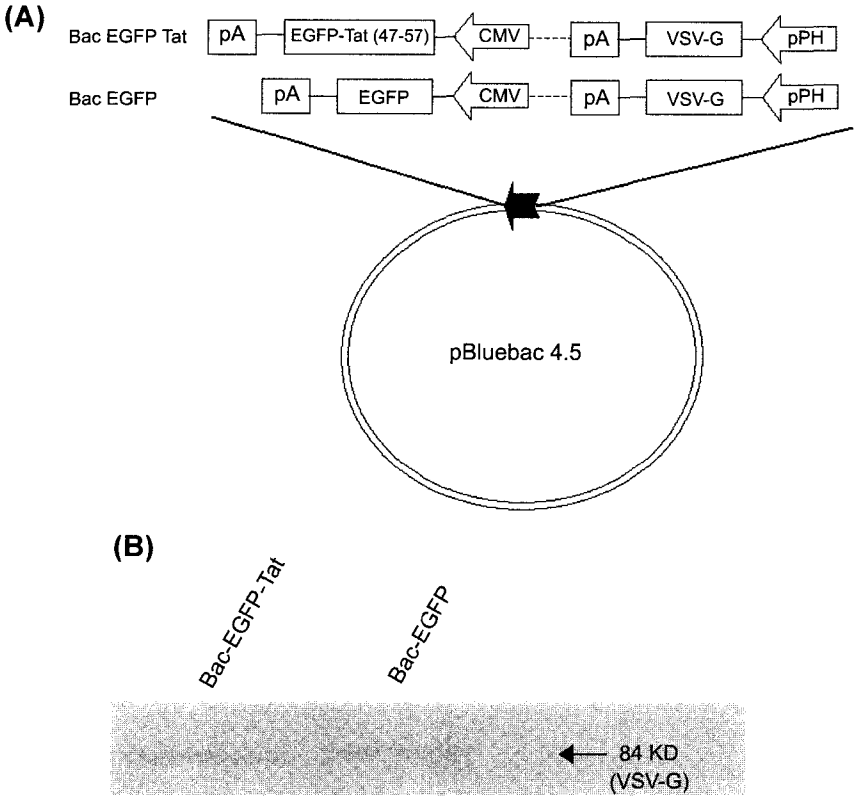

Fig. 3. (A) Construction of baculovirus transfer plasmids. Recombinant baculovirus transfer plasmids containing a VSV-G gene under the control of an Ac*M*NPV polyhedrin (PH) promoter. (B) Western blot analysis of the VSV-G protein induction in Sf9 cells infected with the recombinant baculovirus. Sf9 cells were infected at an m.o.i. of 10, harvested 48 h postinfection, and then examined for VSV-G protein expression using mAb.

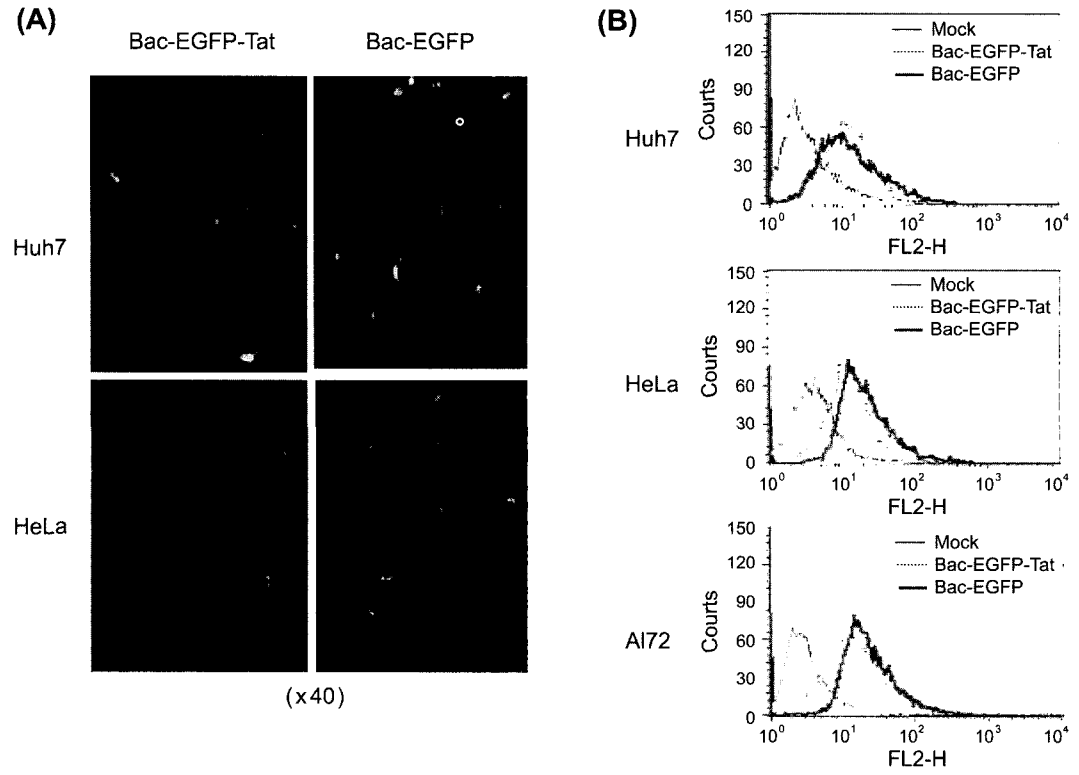

Fig. 4. Intercellular transport ability of EGFP- Tat in infected mammalian cells. (A) Huh7 and HeLa cells were infected with Bac-EGFP-Tat and Bac-EGFP at m.o.i 10. Forty eight hour postinfection the EGFP was detected by fluorescence microscopy. (B) FACS of Huh7, Hela and A173cells infected by recombinant baculoviruses; Bac-EGFP-Tat and Bac-EGFP 48h postinfection. FACS analyses were performed in triplicate.

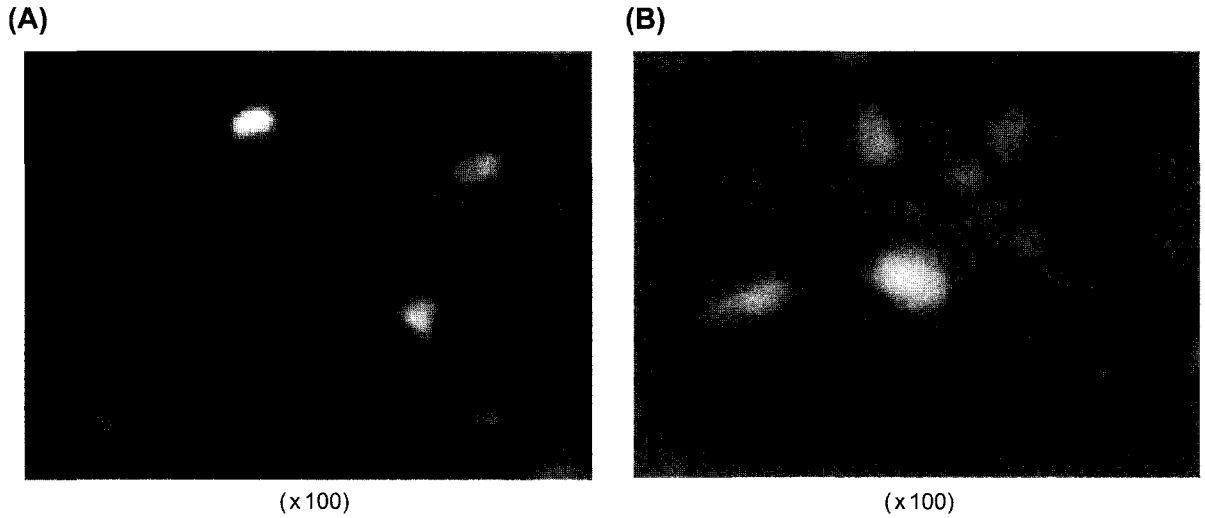

Fig. 5. Tat dependent expression of EGFP in methanol fixed cells. Huh7 cells were infected with (A) Bac-EGFP and (B) Bac-EGFP-Tat (recombinant baculovirus), with the cells fixed in methanol 48 h later.

fluorescence detectable in almost all of the cells (Fig. 5).

Discussion

A number of studies have shown the utility of the HIV-1 Tat protein in gene therapy. This approach has significant capacity for the utility of extracellular proteins; gain of entry to the cell cytosol is not unusual. The discovery of proteins that can apparently translocate across the cell membranes in the absence of specific receptors makes their use for enhanced delivery of attached proteins into cells possible (Leifert *et al.*, 2002). The potential utility of PTDs was clearly demonstrated when the HIV-1 Tat was attached to the bacterial enzyme, β-galactosidase, and the

injection of the fusion product into mice resulted in the expression of enzyme activity in many tissues, including the brain (Schwarze *et al.*, 1999). Widespread β-galactosidase expression was dependent on the attachment of the Tat sequence, indicating that Tat was required for membrane translocation, and could even take a large (~120 kDa) fusion protein across the normally impervious blood brain barrier. However, recent studies have reported that there was no evidence for this ability with HIV-1 Tat. Almost all of these studies added an exogenously prepared Tat fusion protein into the cells, either in culture or *in vivo*. With this in mind, the aim of this study was to determine the ability of the Tat fusion protein using endogenously (denatured form) and exogenously (native denatured form) prepared proteins.

This study confirmed the utility of HIV-1 Tat, as demonstrated by the transduction efficiency of the exogenously prepared Tat fusion EGFP in cell lines using fluorescence microscopy and FACS analysis (Fig. 2). It was found that Tat had a significant effect on the transduction and intercellular localization properties. Using these results, it was concluded that the exogenously added Tat was sufficient for the protein transduction of EGFP across plasma membranes. A Baculovirus mediated-expression-HIV-1 Tat-fusion, with the indicator protein, EGFP, was generated in order to directly evaluate Tat, which was mediated by the intercellular protein transfer in living cells. Careful quantitation of the infection of the cell with a virus encoding EGFP, both with and without the attached Tat, showed no significant difference in the percentage of cells expressing the marker protein, suggesting that no extensive intercellular transfer of Tat-EGFP had taken place (Fig. 4). All of our FACS and confocal analyses were performed on live cells.

Some studies have suggested that intercellular trafficking is an artifact of fixation, and can only be observed after fixation, which strongly suggests that the apparent translocatory effect of Tat may be an artifact of fixation, where the fixative strips away the cell membrane, releasing the PTDs from the transfected cells. Consequently, the liberated protein binds to the exposed nuclei of adjacent cells due to its strong nuclear localization signal (Leifert *et al.*, 2002). A similar effect was observed following methanol fixation of the cells infected with the virus, and the resulting ubiquitous fluorescence depended on the presence of Tat (Fig. 5).

Therefore, it has been shown that endogenously synthesized Tat fusion peptides exhibit no intercellular transport properties in several cell lines. Instead, Tat would enhance the binding of the free protein to the cell membrane, which would facilitate subsequent entry into the cell. Therefore, these results suggest that the Tat passes through the plasma membrane in a single direction. One would predict that these apparently contradictory results, i.e. Tat-β-galactosidase appear to increase the biodistribution, are most likely due to a combination of the lysosomal enzyme's intrinsic transporting properties as well as the protein transduction properties of Tat. Although the ability of Tat is restricted, further studies on proteins other than the EGFP will be needed to determine the value of Tat in gene therapy.

Acknowledgment

This work was supported by a Korea Research Foundation Grant (KRF-2001-005-F20009).

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