U.S. Army Medical Research
Institute of Chemical Defense

USAMRICD-TR-06-01

Expression and Cellular Internalization of Two Tat-conjugated Fluorescent Proteins

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April 2006

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
April 2006	Technical Report	

4. TITLE AND SUBTITLE
Expression and Cellular Internalization of Two Tat-conjugated Fluorescent Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
61101A

6. AUTHOR(S)
Apland, JP, Kincaid, R, Oyler, G, Adler, M

5d. PROJECT NUMBER

5e. TASK NUMBER
91C

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDT-N
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-06-01

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDA-T
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
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15. SUBJECT TERMS
Tat, fusion proteins, YFP, yellow fluorescent protein, cellular internalization, protein translocation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			James P. Apland
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	11	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-5114

ACKNOWLEDGEMENTS

We thank Dr. Heidi Hoard-Fruchey for valuable suggestions and CPT Angela Purcell for assistance with confocal microscopy.

ABSTRACT

Two hybrid vectors were designed for the expression in *E. coli* of fluorescent fusion proteins containing the protein translocation domain designated as Tat. The Tat domain was introduced to promote the entry of cargo protein, in this case the fluorophore yellow fluorescent protein (YFP), into cells. The first construct was made by fusing Tat with YFP. The second Tat fusion protein was constructed to contain YFP and the palmitoylation domain (Palm) from SNAP-25. The Palm domain was intended to bind the fusion protein to intracellular membranes and trap the fluorophore inside the cells. Intracellular localization of both proteins was demonstrated by laser confocal microscopy. This research serves as proof of the concept that such Tat fusion constructs may be useful in intracellular delivery of proteins and drugs that normally cannot penetrate the cell membrane and that the Tat domain remains functional with an intracellular palmitoylation trapping domain present.

INTRODUCTION

To date, the most potent inhibitors of the catalytic activity of botulinum neurotoxin have been small peptides or pseudopeptides (Anne *et al*., 2003). While these peptides are highly effective in cell-free enzymatic assays, they lack *in vivo* efficacy due their inability to penetrate plasma membranes (Simpson, 2004). One way to overcome this difficulty is to couple potential therapeutic agents to transport molecules. A project was initiated in which yellow fluorescent protein (YFP), a derivative of the green fluorescent protein (GFP) obtained from the jellyfish *Aequorea aequorea* (Chalfie and Kain, 1998), was attached to a carrier protein and introduced into living cells as a mimic for a therapeutic agent. YFP has brighter emission at a longer wavelength than the more conventionally used GFP (Heim and Tsien, 1996), which makes it a better intracellular marker. YFP introduction could be achieved via a protein translocation domain within the Tat protein of HIV-1 (Fittipaldi and Giacca, 2005; Zhao and Weissleder, 2004). To preclude possible egress of this fluorescent indicator from neurons, it was also proposed that a "sequestration domain," corresponding to a palmitoylation domain (Palm) from human SNAP-25 (Oyler *et al.*, 1989), could be appended to this protein. Such a modification of the fusion protein might enable the fluorophore, YFP, to be bound to intracellular membranes, thus facilitating its retention inside cells.

In the experiments described in this report, expression of these mammalian proteins in a heterologous *E. coli* system was conducted as a "proof-of-concept" study regarding the feasibility of using similar carrier proteins to introduce therapeutic agents into cells. Cultured NS-26 neuroblastoma cells were exposed to the chimeric proteins to determine whether Tat promoted translocation of YFP into cells. The results suggest that both Tat-YFP and Tat-Palm-YFP were taken up into NS-26 cells and were not readily removed by washing with Hanks buffered salt solution (HBSS).

MATERIALS AND METHODS

Construction of pET22b-HTat-YFP and pET22b-HTat-Palm-YFP vectors

Synthetic cassettes corresponding to a 6 His-Tat and to a 6 His-Tat-Palm domain were designed for expression in vector pET-22. The affinity tag of 6 histidines (His tag) was added to the amino terminus of the proteins to allow purification using nickel-nitriloacetic acid (NTA) affinity chromatography. The coding region for the 11-residue Tat (underlined) was separated from the His-tag and YFP components by di-glycine linkers (blue) as shown: MHHHHHHGG YGRKKRRQRRRGGSMV-YFP. The coding region for the Tat-YFP construct containing Palm (bold) was likewise separated from the His-tag and YFP components by di-glycine linkers: MHHHHHHGGYGRKKRRQRRRGG**LGKCCGLFICPCNKLKSSDYKKAW**GG

SMD-YFP

The protein coding regions were reverse translated utilizing optimal codon usage for expression in *E. coli.* From the optimized DNA sequences, two pairs of oligonucleotides were constructed to have 12-15 nucleotide complementary overlaps. By amplification and extension of oligonucleotide pairs, two PCR products (~82 bp for 6 His-Tat-YFP and ~163 bp for 6 His-Tat-Palm-YFP) were produced. The PCR products encoding these synthetic cassettes were

subcloned into a TA cloning vector (pCR 2.1 topo, Invitrogen Corp., Carlsbad, CA). Competent host cells (TOP 10 supercompetent cells, Invitrogen) were transformed with the ligation reaction, plated onto antibiotic-selection plates, and incubated overnight at 37°C. Isolated colonies of transformants were grown for plasmid preparation to identify a clone having the desired sequence and lacking mutations.

DNA from a sequence-verified pCR 2.1 clone was digested with **Nde I** and **Nco I** restriction enzymes (New England Biolabs, Ipswich, MA), and the fragments corresponding to the synthetic cassettes (~76 bp and ~157 bp) were separated on preparative 2% agarose gels to allow for their excision and chromatographic purification (QIAquick spin columns, Qiagen Inc., Valencia, CA). The destination expression vector (pET22b-EYFP) was prepared by digestion with **Nde I** and **Nco I** followed by purification on agarose gels. Ligation of the purified inserts and linearized pET-22b-EYFP vector was carried out by using T4 DNA ligase (New England Biolabs), and the ligation reaction was transformed into TOP10 *E. coli* followed by antibiotic selection. Several transformants were grown and confirmed by sequencing. Clones having the correct expression sequence were grown for semi-preparative DNA purification. Protein samples were expressed in the vector pET22b using the expression host BL21 (DE3) pLYS and utilizing a soluble fraction purification scheme. Samples were dissolved in Tris-buffered saline solution containing 20% glycerol.

Cell Culture

Clonal NS-26 neuroblastoma cells were obtained from Dr. Marshall Nirenberg (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) as frozen stocks at passage 12. Cells (5×10^4 cells/ml) were thawed and cultured in 35 mm dishes at 37°C in Dulbecco's Modified Eagle's Medium with 5% fetal bovine serum in a humidified atmosphere of 95% air/5% CO_2. Cells were differentiated by addition of dibutyryl cyclic AMP for 7-9 days prior to experimentation. For staining with 6 His-Tat-YFP or 6 His-Tat-Palm-YFP, growth medium was removed and cells were incubated overnight at 37°C in HBSS containing 0.5 µg/ml of 6 His-Tat-YFP or His-Tat-Palm-YFP. Cells were washed three times with dye-free HBSS prior to viewing. All cell culture reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Laser Confocal Microscopy

Cells were imaged with a Nikon C1 confocal microscope (Nikon Instruments Inc, Melville, NY) using a 40X /0.60 NA objective. Images were collected using the Nikon C1 software. The fluorochrome was excited with the 488-nm line of an argon laser and detected using a 515-530 nm band pass emission filter. Fluorescent and brightfield images were obtained as averages of ten scans and superimposed.

RESULTS

A custom YFP destination vector was constructed to enable the cloning of 6 His fusion proteins containing either Tat-YFP or the Tat-Palm-YFP fluorescent indicator elements. After validating the constructs, small expression studies were carried out to determine whether soluble fusion proteins were produced. These studies were then extended by using scaled-up bacterial preparations and purification of the YFP fusion proteins.

Figure 1 shows an immunoblot analysis of protein fractions following Ni^{2+} column purification. These data indicate that the fluorescent proteins are suitable for use in cell culture experiments. The identity of the proteins was confirmed by Western blot analysis (data not shown). Table 1 shows quantities of the various protein fractions generated, as measured by the Bradford assay (Bio-Rad Labs, Hercules, CA). Analysis of these results indicates a low yield with the pET based vector used. However, the yields were sufficient for the pilot cell culture experiments described below.

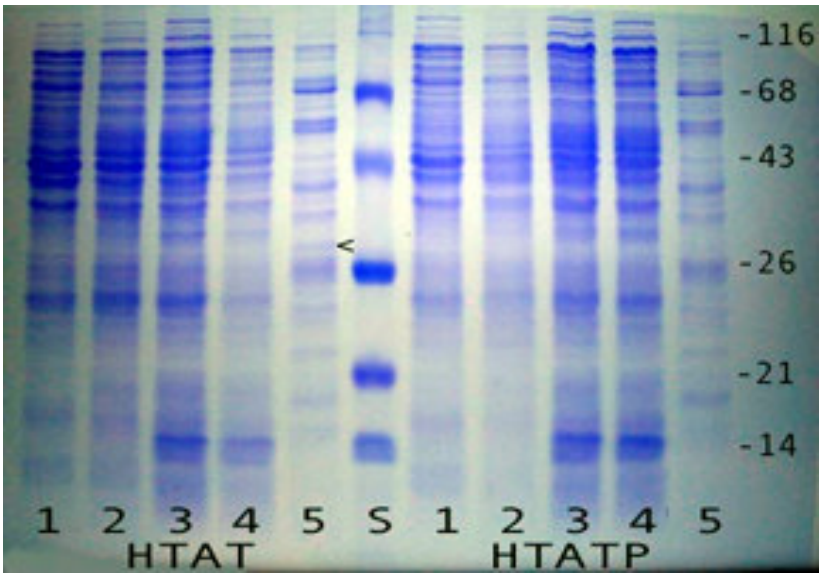

Figure 1. SDS gel and immunoblot analysis of chromatography fractions following Ni^{2+} column purification. Upper panel: Coommassie-stained gel of chromatography fractions; 15 µg of crude fractions and 3 µg of purified fractions were loaded. Molecular weight standards were: 116 kDa, 68 kDa, 43 kDa, 26 kDa, and 21 kDa. Left lanes: 6 His-Tat-YFP (HTAT). Lane 1: HTAT cells, prior to IPTG induction (Total Protein); Lane 2: HTAT cells, after IPTG induction for 3h (Total Protein); Lane 3: HTAT original supernatant; Lane 4: HTAT nickel nitriloacetic acid (NTA) flow-through fraction; Lane 5: HTAT NTA eluate (caret). Lane S: Protein Standard. Right lanes: 6 His-Tat-Palm-YFP (HTATP). Lane 1: HTATP Pre-Induced Cells (Total Protein); Lane 2: HTATP Induced Cells (Total Protein); Lane 3: HTATP Original supernatant; Lane 4: HTATP NTA flow-through fraction; Lane 5: HTATP NTA eluate (faint band at level of caret in lane 5).

Table 1. Protein Estimates on Chromatography Fractions (one column purification)

	Volume (ml)	Conc (mg /ml)
1) HTAT Cells prior to induction (Total Protein)	N/A	0.22
2)HTAT Cells after IPTG induction (Total Protein)	N/A	0.59
3) HTAT Original super ("Or")	0.22	12.5
4) HTAT NTA pass-through fraction ("PT")	3.0	12.5
5) HTAT NTA eluate ("El")	3.0	11.0
6) Protein Standard	-----	-----
7) HTAT-P Pre-Induced Cells (Total Protein)	N/A	0.33
8) HTAT-P Induced Cells (Total Protein)	N/A	0.24
9) HTAT-P Original super ("Or")	3.0	13.0
10) HTAT-P NTA pass-through fraction ("PT")	3.0	13.0
11) HTAT-P NTA eluate ("El")	3.0	13.0

Cultured NS-26 cells were exposed to 6 His-Tat-YFP and 6 His-Tat-Palm-YFP for various times to demonstrate uptake into cells. A maximum concentration of 0.5 µg/ml was selected for each construct, since higher concentrations led to retraction of processes and rounding of cells (data not shown). A wide range of incubation times with the fluorescent constructs was examined. Exposure times of less than 4 hr led to little detectible cellular fluorescence, so it was necessary to extend incubations to 24 hr for most cells to become stained.

Typical micrographs of cultures incubated for 24 hr in the presence of 0.5 µg/ml 6 His-Tat-YFP and 6 His-Tat-Palm-YFP are shown in Figure. 2. Both constructs were taken up by the cells, which maintained normal morphology and no loss of adhesion to the substrate. Cells exposed to 6 His-Tat-YFP showed a consistently brighter fluorescence (Fig. 2A) than those exposed to 6 His-Tat-Palm-YFP (Fig. 2B). This may be due to the higher proportion of YFP in the former construct, which does not include Palm. For both constructs, the staining appeared to be diffuse, and no selective association with organelles or membranes was evident. Interestingly, neither fluorophore was removed by repeated washes or even by overnight incubation in dye-free HBSS (data not shown). Comparison of Figure 2A and Figure 2B reveals that inclusion of the Palm domain is not required for dye-trapping and did not confer selective membrane localization to the 6 His-Tat-Palm-YFP probe. The absence of a demonstrable action of Palm may be due to redistribution of internalized dye over the long incubations times that were required to elicit uptake or to lack of affinity for the plasma membranes.

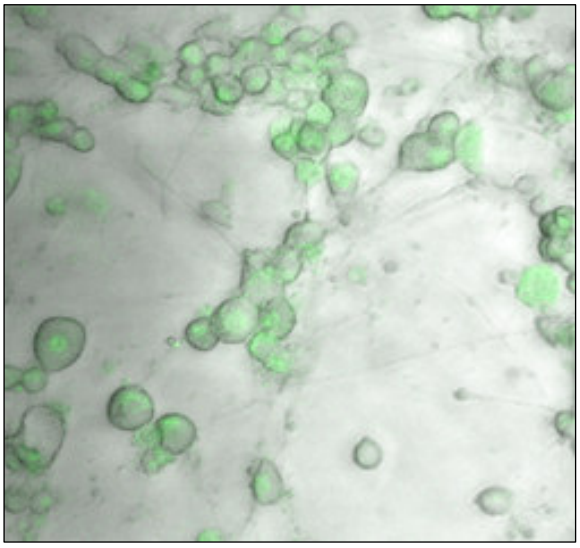

Figure 2A

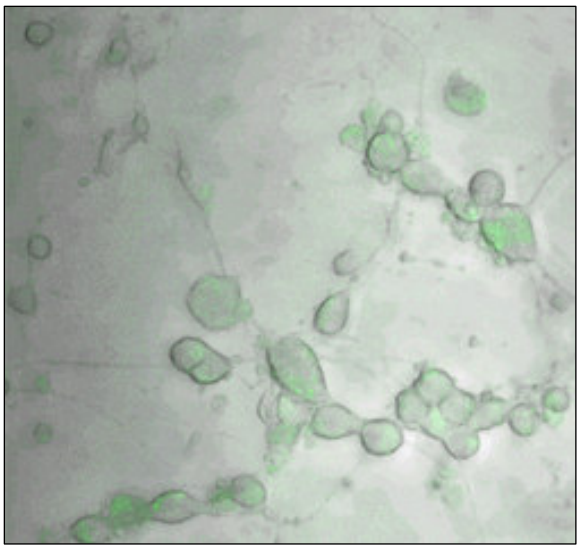

Figure 2B

Figure 2. Laser confocal images of differentiated NS26 cells exposed to chimeric proteins at a concentration of 0.5 µg/ml for 24 hr. Fluorescent images were superimposed on brightfield images (averages of ten scans each). Similar results were observed in 6 additional dishes. No specific pattern of intracellular localization was observed.

A. Cells exposed to 6 His-Tat-YFP. The fluorophore was retained in the cells after extensive washing.

B. Cells exposed to 6 His-Tat-Palm-YFP. The distribution of the fluorophore was similar to that of 6 His-Tat-YFP except for a slightly lower intensity.

DISCUSSION

The results of this pilot study indicate that both chimeric proteins, 6 His-Tat-YFP and 6 His-Tat-Palm-YFP, were taken up by NS-26 neuroblastoma cells. It was expected that 6 His-Tat-Palm-YFP would be palmitoylated and trapped more extensively in the cells than 6 His-Tat-YFP. These results suggest that Palm domain was not extensively bound to intracellular membranes. Furthermore, neither chimeric fluorescent protein was removed by repeated washes. These findings demonstrate that the probes were indeed internalized and not simply deposited on the cell surface. Internalization was presumably achieved by coupling of YFP with the HIV-1-derived Tat protein (Milani et al., 1993) since the YFP molecule by itself is not known to be internalized. Tat proteins appear to be useful in cellular transport of cargo molecules such as botulinum toxin antagonists or indicator probes such as Cameleon YC2.1, a calcium-sensitive FRET fluorophore (Miyawaki *et al.*, 1999). Internalization of these probes was the ultimate goal of the proposed study following validation of the chimeric proteins produced in this project.

A significant limitation of this project was that only small quantities of protein were expressed in these initial "proof of concept" experiments. Low expression may suggest toxicity of the recombinant protein or insolubility of the protein products, neither of which appeared to be the problem in this case. Because little protein was evident in the "induced" samples, it would appear that inefficient translation complexes were formed on ribosomes using these constructs. To circumvent this type of problem, studies using alternative expression vectors and designs will be needed.

CONCLUSIONS AND/OR RECOMMENDATIONS

Additional work is required to increase the yield of protein before production of the indicator fusion protein could be attempted. The 6 His-Tat-YFP and 6 His-Tat-Palm-YFP chimeric proteins are potential vehicles for transporting material into cells, and the Palm domain is, in principle, a potentially effective means of directing the cargo protein to membrane compartments since Palm would be expected to bind to intracellular membranes. However, selective membrane localization could not be demonstrated in this study due presumably to redistribution of internalized dye over the 24-hour period of uptake or to the low purification yield.

REFERENCES

Anne, C, Turcaud, S, Quancard, J, Teffo, F, Meudal, H, Fournie-Zaluski, MC, and Roques, BP. 2003. Development of potent inhibitors of botulinum neurotoxin type B. J. Med. Chem. 46: 4648-4656.

Chalfie, M and Kain, S. 1998. Green Fluorescent Protein. Wiley-Liss, New York.

Fittipaldi, A and Giacca, M. 2005. Transcellular protein transduction using the Tat protein of HIV-1. Adv. Drug Deliv. Rev. 57: 597-608.

Heim, R and Tsien, RY. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. 6: 178-182.

Milani, D, Zauli, G, Neri, LM, Marchisio, M, Previati, M, and Capitani, S. 1993. Influence of the human immunodeficiency virus type 1 Tat protein on the proliferation and differentiation of PC12 rat pheochromocytoma cells. J. Gen. Virol. 74: 2587-2594.

Miyawaki, A, Griesbeck, O, Heim, R, and Tsien, RY. 1999. Dynamic and quantitative calcium measurements using improved chameleons. Proc. Natl. Acad. Sci., USA 96: 2135-2140.

Oyler, GA, Higgins, GA, Hart, RA, Battenberg, E, Billingsley, M, Bloom, FE, and Wilson, MC. 1989. The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J. Cell Biol. 109: 3039-3052.

Simpson, LL. 2004. Identification of the major steps in botulinum toxin action. Annu. Rev. Pharmacol. Toxicol. 44: 167-193.

Zhao, M and Weissleder, R. 2004. Intracellular cargo delivery using Tat peptide and derivatives. Med. Res. Rev. 24: 1-12.