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Technical Note

HIV TAT variants differentially influence the production of glucocerebrosidase in *Sf9* cells

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ABSTRACT. Gaucher disease, the most common lysosomal storage disorder, is currently treated with enzyme replacement therapy. This approach, however, is ineffective in altering the progression of neurodegeneration in type 2 and type 3 patients due to the difficulty of transferring the recombinant enzyme across the blood-brain barrier. Human immunodeficiency virus type 1 *trans*-activating transcriptional activator protein (HIV TAT) contains a protein transduction domain that can be added to a fusion protein partner to allow for transport of the partner across membranes. Consequently, we examined the creation, production, and secretion of fusion constructs containing glucocerebrosidase and either wild-type TAT or modified TAT in *Sf9* cells. All three constructs exhibited successful expression, with wild-type TAT chimeras showing lower levels of expression than modified TAT chimeras.

Key words: HIV TAT, Glucocerebrosidase, Gaucher, Transduction, Protein expression

INTRODUCTION

Gaucher disease, the most common lysosomal storage disorder, is characterized by a deficiency in the enzyme glucocerebrosidase (GBA), which is involved in the natural recycling of membrane sphingolipids (Beutler and Grabowski, 2001). Hallmark symptoms include hepatosplenomegaly, bone crises, and pancytopenia. Three major types of the disease have been classified, based on the absence/presence and severity of primary nervous system involvement: type 1 (non-neuronopathic), type 2 (acute neuronopathic) and type 3 (subacute neuronopathic) (de Fost et al., 2003). Currently, enzyme replacement therapy is the standard in Gaucher disease treatment. This treatment, however, has not been effective in altering the progression of neurodegeneration in type 2 and type 3 patients (Grabowski et al., 1998). Emerging research demonstrating the therapeutic potential of cell-penetrating peptides offers hope for easing neurodegeneration. One cell-penetrating peptide of recent interest is the 86-amino-acid human immunodeficiency virus type 1 *trans*-activating transcriptional activator protein (HIV TAT). HIV TAT contains a protein transduction domain extending from amino acid residues 47-57 that can be added to a fusion protein partner to allow for transport of the partner into cells (Vives et al., 1997; Ho et al., 2001; Ziegler et al., 2005). Recently, a modified version of the HIV TAT protein transduction domain (modTAT), which can confer a 5- to 33-fold increase in transduction ability, was engineered (Ho et al., 2001). However, the influence of the structural modification on fusion protein expression has not been reported. Therefore, we examined the creation, production, and secretion of GBA, TAT•GBA, and modTAT•GBA in *Sf9* cells. All three constructs exhibited successful expression; to our knowledge, this is the first successful production of TAT/GBA chimerae. The addition of wild-type TAT resulted in a lower level of expression than that of modTAT.

MATERIAL AND METHODS

Construct creation

To create the GBA, TAT•GBA, and modTAT•GBA constructs, GBA cDNA was PCR-amplified with the following primers: primer 1 (sense) 5'-TATGAATTCGCCCGCCCC TGCATCCCT-3' and primer 2 (antisense) 5'-GCGGGAATTCTTTAATGCCCAGGCTGAG CC-3' for GBA; primer 3 (sense) 5'-CGAATTCTACGGCCGCAAGAAACGCCGCCAGC GCCGCCGCGGTGGAGCCCGCCCCTGCAT-3' and primer 2 (antisense) for TAT•GBA; primer 4 (sense) 5'-CGAATTCTACGCCCGCGCGGCAGCCCGCCAGGCACGCGCAGGT GGAGCCCGCCCCTGCAT-3' and primer 2 (antisense) for modTAT•GBA. PCR amplification was performed with an initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 1.5 min, 50-60°C annealing gradient for 1.5 min, and 72°C for 2 min, with a final elongation at 72°C for 5 min. Reactions were carried out with 100 ng GBA template, 1X *Pfu* buffer, 0.25 mM dNTPs, 0.6 µM sense and antisense primers, and 0.5 units *Pfu* in a total volume of 50 µL. These amplified cDNAs were digested with *Eco*R1 and ligated into the vector p2ZoptcxF (donation from Dr. T. Pfeifer) prior to electroporation into bacterial cells. Transformants were selected by resistance to Zeocin™ (0.1 mg/mL; Invitrogen, Burlington, ON, Canada) on low salt Luria Bertani plates (pH 7.5). Clones were confirmed by PCR amplification and sequenced to ensure the fidelity of the PCR and cloning steps.

Protein expression and analysis

True positive GBA, TAT•GBA, and modTAT•GBA plasmids were isolated, purified and introduced into *Sf9* cells via transfection with Cellfectin reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. Stable polyclonal cultures were selected by Zeocin resistance (1 mg/mL) and assayed for plasmid incorporation by standard PCR analysis and RT-PCR prior to scale-up to 60-mL shaker flasks. At 120 h post-scale-up, 5 x 10^6 cells were harvested and centrifuged at 200 *g* to separate cells from medium. Medium was collected and concentrated according to the manufacturer's instructions in a 30,000-molecular weight Nanosep centrifugal device (Pall Corporation, East Hills, NY, USA). The cell pellet was lysed (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF), vortex-mixed for 2 min, and centrifuged at 14,000 *g* for 5 min. The resulting supernatant was collected as the cytoplasmic fraction and the pellet was resuspended in 1X PBS as the membrane fraction. Samples were then analyzed by SDS-PAGE, followed by Western blotting (GBA antibody AA16B3, donation from Dr. E. Beutler).

RESULTS AND DISCUSSION

GBA, TAT•GBA, and modTAT•GBA were successfully transcribed (Figure 1) and translated (Figure 2) in *Sf9* cells. All fusion proteins were detected in the medium, rather than in the cytoplasmic or membrane fractions. This was expected due to the presence of the human transferrin signal in the p2ZoptcxF vector, easing the first step of protein purification. Fusion to modTAT resulted in levels of expression comparable to those of GBA alone. In contrast, fusion to wild-type TAT resulted in significantly decreased production relative to both GBA and modTAT•GBA (Figure 2). Crude GBA activity assays (N = 6) using the fluorogenic substrate 4-methyl-umbelliferyl glucopyranoside (4MUGP) were difficult to interpret due to high background levels; however, total activity (measured in nmol h^{-1} mg total $protein^{-1}$) was highest in modTAT•GBA (1509 ± 124), followed by GBA (1226 ± 60), then by TAT•GBA (1009 ± 69).

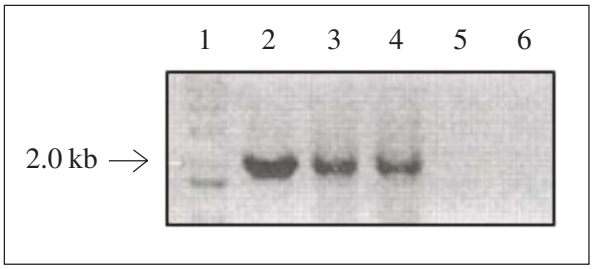

Figure 1. RT-PCR demonstrating successful transcription of glucocerebrosidase (GBA), TAT•GBA, and modTAT•GBA in *Sf9* cells. The procedure was performed using a Superscript kit (Invitrogen, Burlington, ON, Canada) with primers 5'-ACCCAAGCTTATGAGGCTCGCCGTG-3' (sense) and primer 2 (antisense) according to the manufacturer's instructions. Lanes 1-6 are as follows: kb ladder, GBA, TAT•GBA, modTAT•GBA, vector control, and *Sf9* cells only control. TAT = *trans*-activating transcriptional activator protein.

Further scale-up for transduction assays, especially in the case of TAT•GBA, may be accomplished by the integration of more than one insert into the *Sf9* genome, followed by selection for high-expressing clones. The creation of HIV TAT•GBA fusion proteins offers the

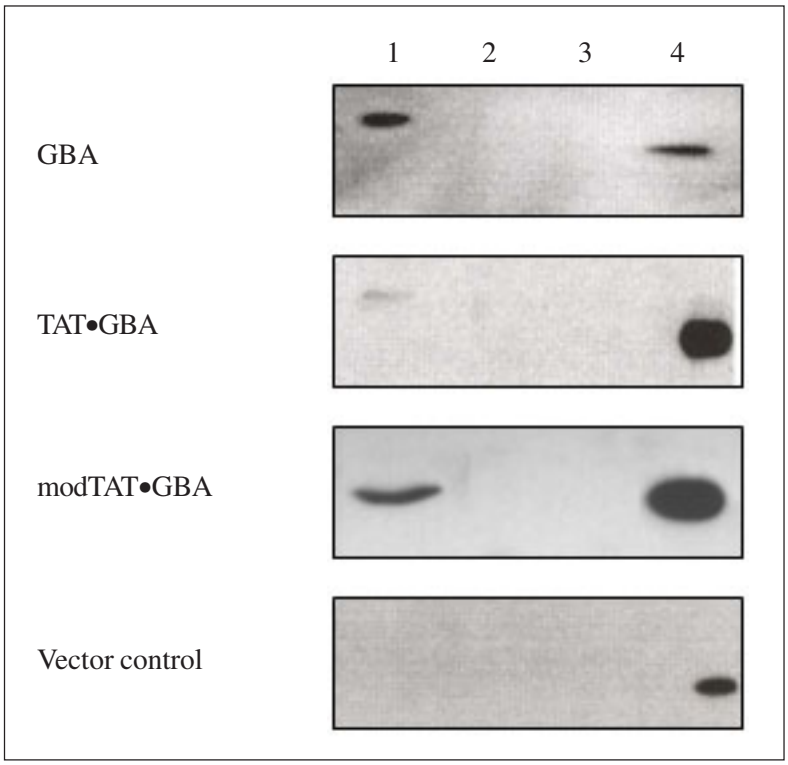

Figure 2. Western blot analysis of glucocerebrosidase (GBA), TAT•GBA, modified TAT•GBA (mod TAT•GBA), and vector control. At 120 h post-scale-up, 5 x 10^6 *Sf9* cells were harvested. Medium, cytoplasmic, and membrane fractions were separated and 10 µg of each were run on a 10% SDS-PAGE gel, followed by immunodetection with anti-GBA antibody. From left to right (lanes 1-4): medium, cytoplasmic fraction, membrane fraction, and GBA positive control. These results were confirmed by three separate experiments. TAT = *trans*-activating transcriptional activator protein.

possibility of utilizing the transduction abilities of HIV TAT to address the treatment of neurodegeneration in Gaucher patients. Since insufficient production levels are often a limiting factor in drug development, researchers should examine the effects of different TAT variants on production as well as on transduction, when considering the engineering of therapy-destined recombinant TAT fusion proteins.

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