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Recombinant GDNF: Tetanus toxin fragment C fusion protein produced from insect cells

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) has potent survival-promoting effects on CNS motor neurons in experimental animals. Its therapeutic efficacy in humans, however, may have been limited by poor bioavailability to the brain and spinal cord. With a view toward improving delivery of GDNF to CNS motor neurons *in vivo*, we generated a recombinant fusion protein comprised of rat GDNF linked to the non-toxic, neuron-binding fragment of tetanus toxin. Recombinant GDNF:TTC produced from insect cells was a soluble homodimer like wild-type GDNF and was bi-functional with respect to GDNF and TTC activity. Like recombinant rat GDNF, the fusion protein increased levels of immunoreactive phosphoAkt in treated NB41A3-hGFRα-1 neuroblastoma cells. Like TTC, GDNF:TTC bound to immobilized ganglioside GT1b *in vitro* with high affinity and selectivity. These results support further testing of recombinant GDNF:TTC as a non-viral vector to improve delivery of GDNF to brain and spinal cord *in vivo*.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor with potent trophic effects on CNS motor neurons *in vitro* [1] and survival-promoting effects on injured motor neurons in experimental animals [2,3]. These experimental findings provided the rationale for a clinical trial of GDNF in patients with amyotrophic lateral sclerosis (ALS) [Amgen clinical trial; Dana C. Hilt, personal communication]. Since GDNF does not cross the blood–brain barrier [4], the protein was administered by intracerebroventricular (ICV) infusion. That GDNF was ineffective in this clinical trial may have been attributable to insufficient access to CNS motor neurons. The poor bioavailability of GDNF in the CNS may also have contributed to the inconsistent results obtained from clinical trials of intraparenchymal GDNF in patients with Parkinson's disease [5].

Tetanus toxin C-fragment (TTC) is the non-toxic neuronal binding fragment of tetanus toxin [6]. Chemical conjugation or recombinant fusion of TTC to various "passenger" proteins has been shown to improve passenger protein delivery to the CNS following intramuscular- [7–9], intraparenchymal- [10], or ICV administration [11]. We have previously reported that chemical conjugation of GDNF to TTC markedly increased levels of GDNF immunoreactivity in adult mouse spinal cord motor neurons following intramuscular injection [12]. To facilitate further evaluation of TTC as a non-viral vector to augment bioavailability of GDNF to the CNS, we engineered a recombinant GDNF:TTC fusion protein in insect cells. This work was prompted by the difficulties encountered generating a GDNF:TTC chemical conjugate, and in trying to obtain soluble GDNF:TTC fusion proteins from bacteria. We report here the successful production of a soluble, bi-functional GDNF:TTC fusion protein from insect cells.

Materials and methods

Production of recombinant baculovirus. Recombinant baculovirus infection of insect cells was used to express two fusion proteins; (1) GDNF:TTC, in which the C-terminus of the mature rat GDNF

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monomer is linked to the N-terminus of TTC; and (2) TTC:GDNF, in which the C-terminus of TTC is linked to the N-terminus of the rat GDNF monomer. A 19-amino acid linker, corresponding to the hinge region of human IgA antibody [13], was engineered into each fusion protein between the GDNF and TTC domains. The cDNA for rat GDNF was obtained from Dr. Ernest Terwilliger (Department of Experimental Medicine, Harvard Institutes of Medicine, Boston, MA, USA). A codon-engineered cDNA for tetanus toxin fragment C was obtained from Dr. Neil Fairweather (Imperial College of Science and Technology, London, UK) [14]. Both fusion proteins were expressed in secreted form behind the honeybee melittin secretion signal using the pMelBac transfer plasmids from InVitrogen (Carlsbad, CA, USA).

Sf21 insect cells (Pharmingen) were cotransfected with a mixture of linearized baculoviral DNA (InVitrogen) and either pMelBac:GDNF:TTC or pMelBac:TTC:GDNF. Single clones of recombinant baculovirus were plaque purified using X-gal staining (200 μg/ml) for detection. High-titer viral stocks (>1×10^8 pfu/ml) were obtained from suspension cultures of Sf9 cells infected at an MOI of 1 and propagated in Sf900-II serum-free media (InVitrogen).

Expression of fusion proteins in insect cells. Fusion proteins were expressed in High Five insect cells (InVitrogen) at 27 °C in Express Five serum-free media (InVitrogen) supplemented with 17 mM L-glutamine and 10 μg/ml gentamicin. Cells were infected at an MOI of 8–10. Conditioned media was harvested when cell viability had decreased to 70–80%. The media was clarified by centrifugation at 10,000*g* for 20 min and concentrated by tangential flow filtration.

Levels of target protein in conditioned media were determined by antigen-capture ELISA using mouse monoclonal anti-GDNF antibody (R and D Systems, 100 ng/0.1 ml/well) as the capture antibody and rabbit anti-TTC antisera (Rockland Immunochemicals, 1:15,000) as the detection antibody. The standard curves for both the GDNF:TTC and TTC:GDNF ELISAs were linear within a concentration range of 0.04–1.25 ng/ml ($r^2 \geqslant 0.99$).

Conditioned media was also evaluated by Western blot analysis using affinity-purified, mouse monoclonal antibody against human GNDF (200 ng/ml, Santa Cruz Biotechnology). Immunoreactive bands were detected with HRP-conjugated donkey anti-mouse IgG (1:20,000, Jackson ImmunoResearch) in conjunction with enhanced chemiluminescence (ECL Plus, GE HealthCare).

Protein purification. Recombinant GDNF:TTC and TTC:GDNF were captured from concentrated insect cell media by cation exchange chromatography on SP Sepharose Fast Flow (GE Healthcare) in batch mode. Approximately 60 ml of resin was incubated overnight at 4 °C with 0.8 L of concentrated media. After washing the resin with 500 ml of 20 mM 2-[*N*-morpholino]ethane-sulfonic acid (MES) buffer, pH 5.8, containing 300 mM NaCl, 1 mM EDTA, 1 μg/ml pepstatin, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF), the target protein was eluted isocratically with 600 mM NaCl in the same buffer. Column fractions enriched in fusion protein were identified by Coomassie Blue staining of SDS–PAGE gels. Pooled fractions were dialyzed against 20 mM Tris–HCl, pH 7.5, containing 100 mM NaCl, 0.5 mM EDTA, and 0.4 mM PMSF and applied to a 5 ml column of Q Sepharose Fast Flow anion exchange resin (GE Healthcare) equilibrated with the dialysis buffer. Both fusion proteins were collected in the column flow-through.

Solid ammonium sulfate was added directly to the Q Sepharose flow-through to a final concentration of 1 M. Samples were loaded onto a hydrophobic interaction chromatography column of 4 ml Butyl Sepharose Fast Flow (GE Healthcare) at room temperature equilibrated with a start buffer of 50 mM K_2HPO_4, pH 7.0, 1 M ammonium sulfate, 1 mM EDTA, and 0.4 mM PMSF (1 ml/min). Bound protein was eluted using steps of 0.9, 0.8, 0.7, 0.6, and 0.5 M ammonium sulfate. The fusion proteins typically eluted in the 0.9 and 0.8 M fractions.

Approximately 3 ml of sample (15–20 mg protein) were loaded on a 16×60 cm Sephacryl S-200 HR gel filtration column (GE Healthcare) equilibrated with 10 mM MES buffer, pH 6.3, containing 300 mM NaCl, 1 mM EDTA, 0.4 mM PMSF and 0.05% Tween 20, and separated at a flow rate of 0.4 ml/min. The column effluent was monitored for absorbance at 280 nm. Fractions enriched in the dimeric form of the fusion proteins were identified by non-reducing SDS–PAGE. Pooled fractions were concentrated with an Amicon Ultra-15 centrifugal filter (100 kDa cutoff), and buffer exchanged into 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, and 0.4 mM PMSF.

Finally, samples were loaded onto a 1 ml HiTrap Heparin HP column (GE Healthcare) equilibrated with the above sodium phosphate buffer. The column was washed with 10 ml of 10 mM sodium phosphate, pH 7.0, containing 400 mM NaCl, and the fusion proteins were eluted using steps of 450, 500, 550, and 600 mM NaCl in the same buffer. GDNF:TTC and TTC:GDNF eluted in the 500- and 550 mM salt fractions. Fractions were pooled, dialyzed against 10 mM Hepes, pH 7.0, containing 150 mM NaCl, filtered through a 0.2 μm syringe filter, frozen on dry ice, and stored at −80 °C.

The solubility of frozen/thawed GDNF:TTC was confirmed by size-exclusion chromatography. One hundred micrograms of protein was loaded on a Superdex 200 column (HR10/30, GE Healthcare) and the column was run at 20 ml/h in 5 mM MES buffer, pH 6.5, containing 300 mM NaCl and 0.05% Tween 20. The column effluent was monitored for absorbance at 280 nm.

Edman degradation sequencing of purified GDNF:TTC and TTC:GDNF was performed on an Applied Biosystems Model 491A protein sequencer equipped with an on-line HPLC system. Protein samples were spotted on a 2×5 mm piece of polyvinylidene difluoride membrane and sequenced directly.

Recombinant HisTTC expressed in *Escherichia* coli was purified from bacterial cell lysates as described previously [11]. Recombinant HisTTC expressed as a secreted protein from insect cells was purified by cation exchange chromatography (see above), immobilized metal affinity chromatography (Nickel Sepharose Fast Flow, GE HealthCare), and gel filtration chromatography (see above).

Protein stability study. The stability of purified GDNF:TTC was examined in sterile vehicle and human CSF. Normal human CSF was obtained from an IRB-approved repository of samples from control- and ALS patients. GDNF:TTC was added to either 10 mM Hepes, pH 7.0, containing 150 mM NaCl, or human CSF at a final concentration of 0.15 mg/ml. Under aseptic conditions, 20 μl aliquots were removed after 1, 3, and 7 days incubation at 37 °C. Stability was assessed by staining of SDS–PAGE gels with Coomassie Blue.

In vitro bioassay for GDNF activity. GDNF activity of recombinant GDNF:TTC was determined by immunoblot analysis of phosphoAkt levels in lysates of treated NB41A3-hGFRα-1 neuroblastoma cells [15]. Recombinant rat GDNF (rrGDNF; R and D Systems) was assayed in parallel as a positive control. Blots were probed with rabbit polyclonal antibody to phosphoAkt (Cell Signaling Technologies, 1:1000) and total Akt (Cell Signaling Technologies, 1:1000).

In vitro binding assay for TTC activity. The neurotropic properties of GDNF:TTC were evaluated using an *in vitro* ganglioside binding assay [16]. Bound ligand was detected with rabbit anti-TTC antisera (Rockland Immunochemicals, 1:10,000).

Results

We used recombinant baculovirus infection of cultured insect cells to produce two soluble fusion proteins, GDNF:TTC and TTC:GDNF, comprised of rat GDNF monomer linked to TTC. The fu-