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Purification of bioactive glycosylated recombinant glial cell line-derived neurotrophic factor

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) neuroprotective effect on dopaminergic neurons has been described in vitro and in vivo, turning up as a promising drug for the treatment of Parkinson's disease. Unglycosylated bacteria-obtained GDNF has already been successfully delivered for a long period of time through an infusion pump directly to the putamen of Parkinsonian patients. Nevertheless, improved distribution and safety issues need to be solved and alternative strategies to long-term delivery seem necessary. The use of glycosylated GDNF could eliminate some safety concerns regarding the presence of antibodies against exogenous unglycosylated GDNF used for the treatment. Therefore, we have chosen a mammalian expression system as a source of glycosylated GDNF. In the present work, we describe the purification of recombinant rat GDNF from the culture media of baby hamster kidney (BHK) cells through several purification steps. Highly pure *N*-glycosylated recombinant GDNF has been obtained similar to the endogenous protein. Furthermore, the purified protein is biologically active when tested its ability to induce PC12 neurite outgrowth.

Keywords: GDNF; Parkinson's disease; Protein purification

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the *sustantia nigra pars compacta* (SNpc). The dopamine failure of the nigrostriatal pathway leads to a profound dopamine deficiency, causing bradykinesia, tremor, rigidity and postural imbalance. The current treatment of PD consists of the pharmacological substitution based on systemic administration of dopamine precursors and agonists. However, following a given period of several years, this symptomatic approach leads to limiting side-effects such as diskinesia and "on-off" phenomenon (Luquin et al., 1992). Although these patients may be helped with surgical therapies such as deep brain stimulation (Rodriguez-Oroz et al., 2004), nowadays it is not possible to halt the progressive loss of the remaining dopamine neurons.

Numerous studies have described the neuroprotective effect of the glial cell line-derived neurotrophic factor (GDNF) on dopaminergic neurons in vitro (Lin et al., 1993; Hou et al., 1996). Protection and restoration of the dopaminergic pathway have been reported with intraparenchymal brain delivery of GDNF on various animal models (Tomac et al., 1995; Gash et al., 1996; Rosenblad et al., 1999). These encouraging results led to two phase I open clinical trials consisting in the intraputamenal administration of GDNF by a pump that resulted in a clear benefit in Parkinsonian patients (Gill et al., 2003; Slevin et al., 2005). However, a randomized, phase II, controlled clinical trial was halted due to the lack of beneficial effects along with safety concerns (Lang et al., 2006). Subsequent data analysis demonstrated that the study was underpowered and thus incapable of ruling out a large effect of GDNF on PD. Therefore the results of the trial should have been described as 'inconclusive' (Hutchinson et al., in press). Differences in dose, catheter design, and delivery protocols may also explain the discrepancy

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF receptor α 1; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction; CM, conditioned medium; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, substantia nigra pars compacta

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between the phase I and II studies. Recombinant GDNF used in all trials was produced in *Escherichia coli*. Bacteria are unable to introduce post-translational modifications, therefore, the protein employed in the trials was unglycosylated. One of the safety concerns was the detection of antibodies against GDNF in blood samples, although all patients remained asymptomatic (Lang et al., 2006). Antibodies were generated against the exogenous GDNF produced in bacteria, which is quite different from the endogenous GDNF. It is not known whether these antibodies neutralize endogenous GDNF activity (Slevin et al., 2006). Furthermore, the use of a mechanical system to deliver GDNF is not exempted from potential risks at implantation, infection or limited diffusion of the drug. Therefore, alternative strategies to long-term delivery of GDNF are necessary.

Drug-releasing biodegradable microspheres loaded with GDNF could represent an alternative strategy. The nature of the GDNF used to load the microparticles is critical, being the glycosylated GDNF the most appropriate. Among the different ways of obtaining a recombinant glycosylated protein, mammalian expression systems offer the greatest degree of product fidelity necessary for a therapeutic agent (Walsh and Jefferis, 2006). The aim of this work was to obtain high amounts of pure bioactive glycosylated GDNF. In this paper we describe the production and purification of GDNF using a mammalian cell system. The purified protein is highly pure, bioactive and glycosylated, and therefore suitable for its microencapsulation within biodegradable microspheres.

2. Materials and methods

2.1. Materials

Recombinant insect cell-derived rat GDNF was purchased from R&D Systems, polyclonal antibody anti-GDNF from Santa Cruz, and BHK and PC12 cells from the ATCC. Tissue culture plasticware was purchased from Corning and, tissue culture media and its additives from Invitrogen. General laboratory reagents were purchased from Sigma unless specified in the text.

2.2. Cell culture

BHK cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Rat PC12 cells were cultured on collagen coated plates (5 $\mu g/cm^2$) in D-MEM supplemented with 5% horse serum, 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin.

2.3. DNA cloning

A cDNA fragment containing the coding region of the rat GDNF gene (GeneBank accession no. L15305) was obtained by reverse transcription (RT)-PCR from rat P1 brain total RNA. Total RNA was extracted using Ultraspec (Bioteck) following manufacturer's instructions including the optional DNase treatment. The cDNA was obtained by incubating 2 μg of total RNA, 1 mM dNTP mix, 3 mM DTT, 5 pM random hexamers, 40 U RNase inhibitor (Promega) and 200 U M-MLV reverse transcriptase (Promega) in a final volume of 30 μl for 1 h at 37 °C. GDNF cDNA was amplified by PCR under the following conditions: 3 μl of cDNA, 0.3 mM dNTP, 1 mM $MgSO_4$, 0.2 μM primers and 2.5 U of platinum/Pfx/DNA polymerase (Invitrogen) in a final volume of 50 μl. Forward oligonucleotide 5′-CTCTAAGATGAAGTTATGGGATG-3′ (from nucleotides 43 to 65) and reverse oligonucleotide 5′-AGGGTCAGATACATCCACACC-3′ (from nucleotides 669 to 689) were used. The PCR fragment was subsequently cloned into pcDNA3.1 (Invitrogen). The identity of the GDNF fragment was confirmed by DNA sequencing. The fragment was then subcloned into the mammalian expression vector pDEST26 by recombination using the Gateway Technology from Invitrogen as described by the manufacturer.

2.4. Production of GDNF

BHK cells were transfected with pDEST26-GDNF using the Lipofectamine Plus reagent according to manufacturer's instructions (Invitrogen) and cell clones stably expressing GDNF were selected by adding 1 mg/ml of G418 (Invitrogen) to the culture medium. G418-resistant clones were isolated on 24-well tissue culture plates and analyzed as follows. Cells were lysated with Ultraspec (Bioteck), total RNA was extracted, retrotranscribed and analyzed by PCR to detect the presence of GDNF mRNA. The culture medium was analyzed by Western blotting to detect the GDNF protein. The clon producing the highest amount of GDNF was expanded and grown in 850 cm^2 roller bottles in the presence of 10% FBS until the cells reached 80% confluence. Afterwards alternating 24 h cycles with culture medium with and without FBS were performed. Conditioned medium harvested in the absence of FBS (50 ml/bottle each cycle) was filtered through a 0.22 μm filter unit and adjusted to pH 7.9 with 5 M NaOH.

2.5. Purification of GDNF

The first step of the purification process consisted in a cation exchange chromatography. The medium was passed through a column loaded with a High Performance SP-Sepharose Fast Flow resin (GE Amersham Biosciences) in 10 mM phosphate buffer pH 7.9, 5 mM EDTA and 150 mM NaCl at 4 °C. Bound proteins were eluted with a linear gradient from 0.15 to 1 M NaCl. Fractions containing GDNF were identified by Western blotting, pooled and concentrated by ultrafiltration through an Amicon device (MW 10 kDa) (Millipore). The second chromatography employed in the purification was a gel filtration. The concentrated sample was then applied to a Superdex 200 HR 10/30 (GE Amersham Biosciences) using an AKTA Purifier system (GE Amersham Biosciences) in 10 mM phosphate buffer pH 7.9 and 150 mM NaCl. Eluted fractions were analyzed by Western blotting. Those containing GDNF were pooled and concentrated through an Amicon device to 1 mg/ml. Aliquots were subjected to SDS-PAGE and the gel stained with GelCode Blue stain reagent (Pierce) or Silver Stain Plus (Bio-Rad). The recombinant GDNF showed a purity

greater than 90%. GDNF protein was quantified by ELISA (Promega).

2.6. Western blot analysis

Protein concentration was determined using Protein Assay (BioRad). SDS-PAGE was performed onto 12.5% polyacrylamide gels, proteins were transferred onto nitrocellulose membranes. Western blotting was performed by incubating the membrane with the blocking solution: 5% non-fat dry milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h, followed by the incubation with anti-GDNF antiserum 1:2000 in blocking buffer for 16 h at 4 °C. After washing with TBST, the blot was incubated in blocking solution with the secondary antibody donkey anti-rabbit 1:2000 (GE Amersham Biosciences), labeled with horseradish peroxidase for 1 h, and detected by chemiluminescence using the Lumi Light Plus Reagent (Roche). Unless specified, all incubations were performed at room temperature.

2.7. PC-12 neurite outgrowth bioassay

The biological activity of GDNF was tested after each purification step as the ability to induce neurite outgrowth on PC12 cells. The expression of the two GDNF receptors, RET and GFRα1, on PC12 cells was confirmed by RT-PCR. Total RNA was isolated from PC12 cells grown on a 60 mm culture plate. Single stranded cDNA was synthesized using MMLV reverse transcriptase and random hexamers as described previously in the "DNA cloning" section. The cDNA was amplified using the following primer sets—RET: forward, 5′-AGGGACGGAAGATGAAGATCTC3-3′; reverse, 5′-AGGTTGAAGAGTCGTTCAGGAG-3′; GFRα1: forward, 5′-ACGACACCTGTAAGAAGTACAG-3′; reverse, 5′-CTCGTTCTTCATAGGAGCACAC-3′. PC-12 cells were plated onto a 12 well culture plate at a low density, 2×10^3 cells/cm^2. Either commercial GDNF or purified GDNF (50 ng/ml) was added 24 h later and neurite outgrowth was visualized after 7 days under phase contrast illumination with a Leika DM IRB inverted microscope connected to a Hamamatsu ORCA-ER digital camera.

2.8. N-Glycanase digestion

Two micrograms of purified GDNF were denatured in 20 mM phosphate pH 7.5, 0.1% SDS and 50 mM β-mercaptoethanol by heating at 100 °C for 5 min. NP-40 (0.75%) and 0.0025 U of *N*-glycosidase F (Prozyme) were added and the reaction was incubated at 37 °C for 6 h. Digested GDNF was analyzed by Western blotting.

3. Results

3.1. Analysis of insect cell-derived rat GDNF

Commercially available recombinant rat GDNF obtained from an insect cell line was analyzed by SDS-PAGE followed by silver staining (Fig. 1A) or Western blot using a polyclonal antiserum anti-GDNF (Fig. 1B). The analysis showed that GDNF migrates as a three band protein due to different glycosylation patterns. The 15 kDa band is the lowest and most abundant isoform of GDNF, and corresponds to the molecular weight of the unglycosylated protein. The other two bands, 20 and 23 kDa, correspond to two different patterns of glycosylation, produced by the insect cells used for the production of the protein. The presence of many other bands visualized when the protein was analyzed by SDS-PAGE and silver stain (Fig. 1A), the possible role of glycosylation, and the high amount of GDNF needed for the preparation of the microparticles, prompted us to develop our own source of neurotrophic factor. The recombinant rat GDNF from the insect cell line was used as a control throughout the purification process.

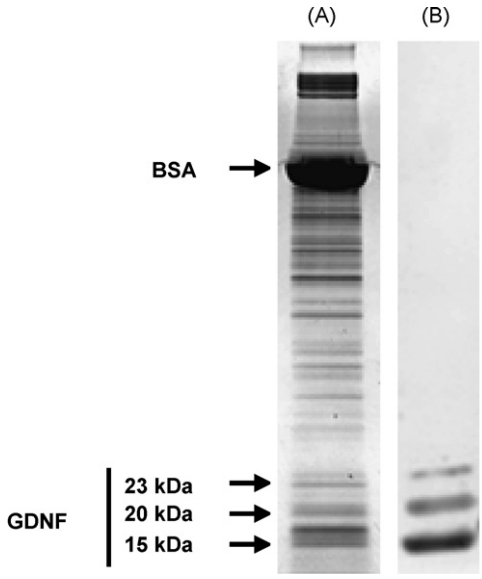

Fig. 1. Visualization of insect cell-derived rat recombinant GDNF. (A) Insect cell-derived GDNF (1 μg) was subjected to SDS-PAGE followed by silver staining under reducing conditions. Arrows indicate BSA used to stabilize the protein and the three bands that correspond to GDNF due to a different pattern of glycosylation. (B) Western blotting of GDNF showing that the antiserum anti-GDNF recognizes the same three isoforms of the protein.

3.2. Purification of GDNF

We had previously cloned the full-length rat GDNF cDNA (644 pb) from rat striatum for in situ hybridization (Barroso-Chinea et al., 2005). Using the Gateway technology and through a couple of recombination reactions the GDNF was transferred, first into a pDONR201 plasmid and then into the mammalian expression vector pDEST26. Cell lines COS-7, 293T and BHK cells were stably transfected, and several clones from each cell line were selected and expanded. The analysis of these clones by PCR revealed that most of them had been successfully transfected and were expressing the mRNA for GDNF. When we studied the amount of protein released to the conditioned medium (CM) (Fig. 2A) by Western blotting (Fig. 2B), BHK cells were the highest GDNF producing cells (data not shown). Therefore, serum free CM from BHK was selected for purifica-

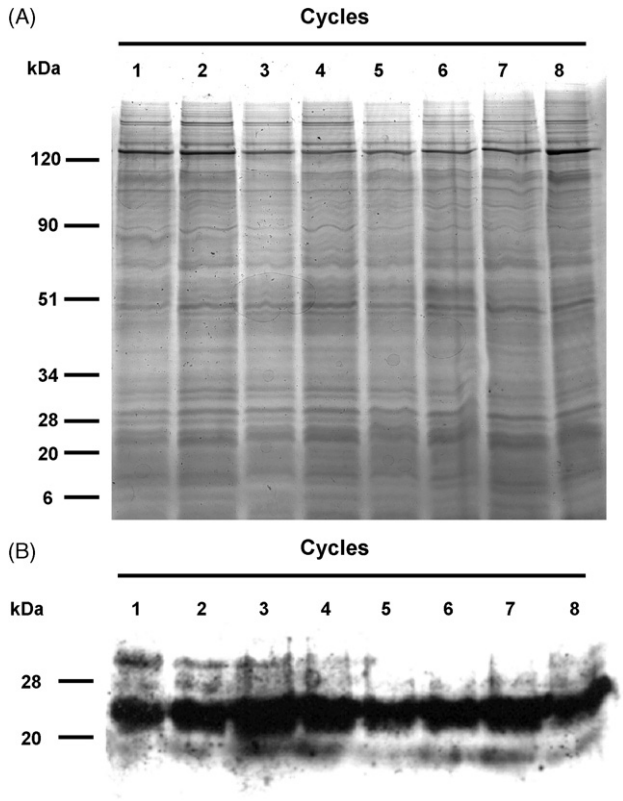

Fig. 2. Conditioned medium from BHK cells transfected with the mammalian expression vector containing the rat GDNF. After transfection with the pDEST26-rGDNF vector, positive clones were selected by their ability to grow in the presence of G418. The highest GDNF-producing clone was expanded and was grown in roller bottles alternating 24 h cycles in the presence or absence of FBS. (A) Silver staining after SDS-PAGE of the conditioned medium collected from each cycle after 24 h incubation without FBS. (B) Western blotting of the conditioned medium from cycles without FBS confirming the release of GDNF to the extracellular medium.

tion. The starting material contained GDNF at 0.25 μg/ml from a total protein concentration of 40 μg/ml.

CM was passed through a 0.22 μm pore diameter filter and loaded onto a SP-Sepharose column at pH 7.9. A gradient of NaCl eluted GDNF at approximately 0.5 M NaCl. The theoretical pI = 9.44 of the protein was in agreement with the fact that GDNF bound tightly to the cation exchange resin at pH 7.9. Fractions were analyzed by SDS-PAGE followed by Coomasie Blue staining (Fig. 3A) and Western blotting (Fig. 3B). Those fractions containing GDNF were pooled and concentrated through an Amicon device. On a subsequent gel filtration chromatography on a Superdex 200, GDNF eluted as a single symmetrical peak (Fig. 4A) corresponding to the GDNF homodimer. Fractions comprising the peak were analyzed by Western blotting (Fig. 4B) under reducing conditions, demonstrating that the peak corresponded to three GDNF isoforms, 20, 23 and 26 kDa, being the 26 kDa form the most abundant one. Again, these fractions containing GDNF were pooled and concentrated to 1 mg/ml. The molecular size of GDNF produced in mammalian cells (Figs. 3B and Fig. 4B) was different from the molecular size of GDNF produced in insect cells (Fig. 1) probably due to a different glycosylation pattern.

3.3. Analysis of purified GDNF

The purity of the final protein sample was assessed by SDS-PAGE and silver staining. As shown in Fig. 5A, GDNF was purified to homogeneity from CM showing a unique protein band with an apparent size of 26 kDa, which was the most abundant form of the monomeric protein when analyzed by Western blotting (Fig. 4B) under reducing conditions. The observed molecular mass of purified GDNF was larger than its predicted molecular weight (15 kDa) and larger than the insect cell-derived GDNF. The aminoacid sequence of GDNF contains several potential glycosylation sites and the appearance of different molecular size bands due to *N*-glycosylation has been previously described (Lin et al., 1994; Trupp et al., 1995). To confirm that

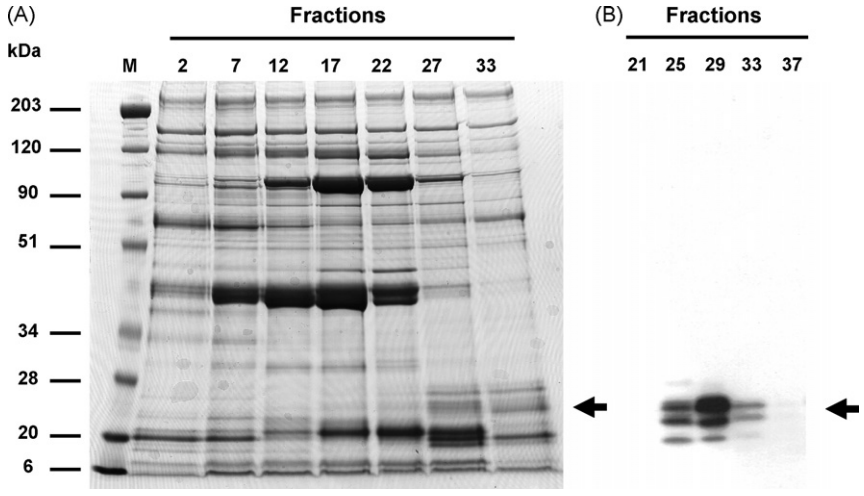

Fig. 3. Cation exchange chromatography. Conditioned medium obtained from BHK-GDNF cells was adjusted to pH 7.9 and then applied to a SP-Sepharose exchange column. Bound GDNF was eluted with a salt gradient from 0.15 to 1 M NaCl. Fractions (2.5 ml) were analyzed by SDS-PAGE under reducing conditions followed by Coomasie Blue staining (A) and Western blotting (B). Arrows show GDNF.

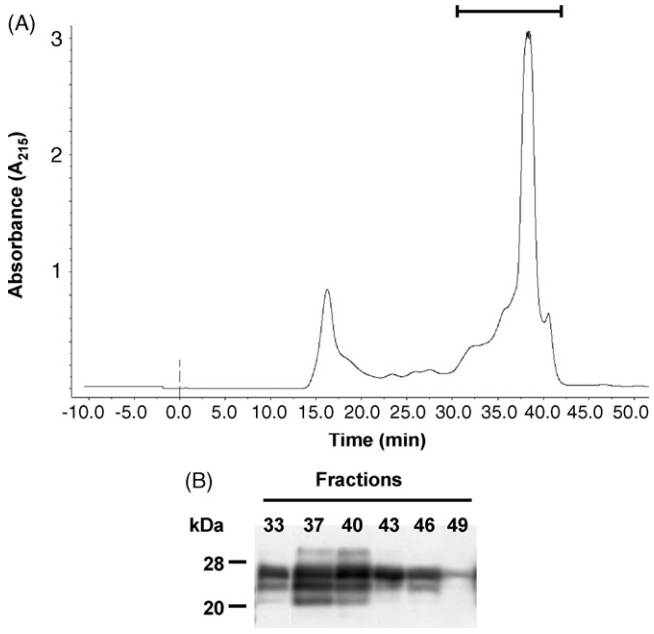

Fig. 4. Gel filtration chromatography. A concentrated pool of fractions from the SP-Sepharose chromatography showing GDNF immunoreactivity was applied to a Superdex 200 HR column and 0.25 ml fractions were collected. (A) Protein profile (absorbance at 215 nm) over time (min) from the gel filtration column chromatography showing a major peak that corresponds to the fractions displaying GDNF immunoreactivity (B). The bar indicates the fractions that were analyzed by Western blotting.

this was the case, purified GDNF was treated with *N*-glycanase (Fig. 5B). Deglycosylation reduced the apparent molecular mass to the predicted 15 kDa. Therefore, the differences in mobility between insect cell-derived GDNF and mammalian cell-derived GDNF are due to carbohydrate linkage and not to the core protein.

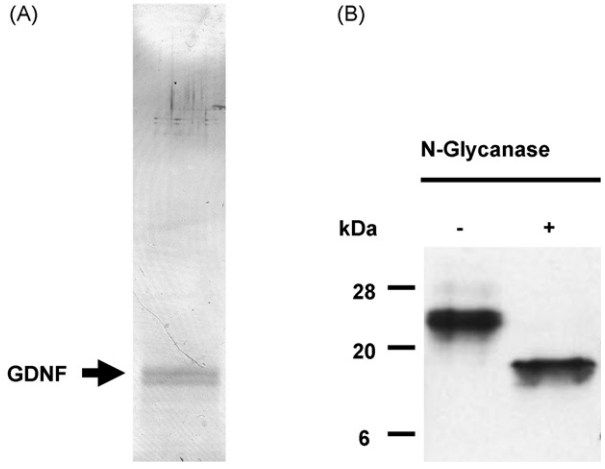

Fig. 5. Analysis of the purified rat recombinant GDNF. (A) The purity of the protein was analyzed by SDS-PAGE under reducing conditions and silver staining of the gel. The arrow shows the main band corresponding to GDNF (26 kDa). (B) *N*-Glycanase treatment of mammalian recombinant GDNF analyzed by SDS-PAGE and Western blotting. In the absence of *N*-glycanase the main band observed has a molecular weight of 26 kDa. *N*-Glycanase treatment results in the disappearance of the 26 kDa and the appearance of a 15 kDa band corresponding to the size of the unglycosylated protein.

3.4. Biological activity

PC12 cells were used to evaluate the activity of the purified GDNF. Induction of PC12 differentiation was chosen because it is an easy and reproducible technique. GDNF exerts its biological action through two receptors: GFRα1 and RET. Whether PC12 cells express both receptors was not clear, since the absence of GFRα1 (Wang et al., 1998) or RET (Powers et al., 2002) in this cell line has been described. Therefore, we first examined the presence of both receptors in PC12 cells by RT-PCR. PC12 cells expressed both receptors (Fig. 6A). We assayed the ability of purified GDNF to induce neurite outgrowth from PC12 cells. As shown in Fig. 6B, control cells displayed a typical undifferentiated morphology. In contrast, striking neurite outgrowth was observed in cultures treated with 50 ng/ml of mammalian cell-derived GDNF or insect cell-derived GDNF. Therefore, GDNF molecules from different origins and with different patterns of glycosylation exerted a similar differentiating activity. These results demonstrate that the protein purified from the BHK-CM exhibited the biological activity of GDNF.

4. Discussion

Results from the phase I and II clinical trials (Gill et al., 2003; Slevin et al., 2005; Lang et al., 2006) have pointed out the need of new strategies for long-term delivery of GDNF directly to the putamen. Microparticles loaded with recombinant GDNF could represent an interesting alternative but, the first and critical step, is to obtain high amounts of pure and bioactive recombinant neurotrophic factor. Among the different systems to produce recombinant proteins, the most suitable for this purpose would be the one producing glycosylated GDNF. Production of recombinant proteins using a prokaryotic bacterial system such as *E. coli*, is the most widely employed because bacteria grow very fast in simple and cheap media. However, there are serious limitations in using bacteria for the production of eukaryotic proteins. Bacterial cells are not capable of carrying out post-translational modifications such as glycosylation. In addition, bacteria have low levels of protein secretion to the extracellular medium and proteins expressed in large amounts often precipitate into insoluble aggregates called inclusion bodies that have to be recovered by solubilisation in denaturing agents followed by careful renaturation. There are other systems of choice to produce glycosylated proteins. Yeast are easy to manipulate, they secrete the protein to the extracellular media and are able to glycosylate proteins. However, their oligosaccharide chains are significantly different from their mammalian counterparts. Baculovirus-infected insect cells used as expression systems also introduce post-translational modification but their glycosylation capability is limited to produce mannose rich oligosaccharides and do not synthesize complex oligosaccharides. Despite many shortcomings, mammalian cells are still the ideal expression system to produce complex-structured proteins, as they offer the greatest degree of product fidelity necessary for a therapeutic agent (Walsh and Jefferis, 2006). Since the final use of the purified protein is to prepare GDNF-loaded microparticles for the treatment of PD, we decided that the appropriate

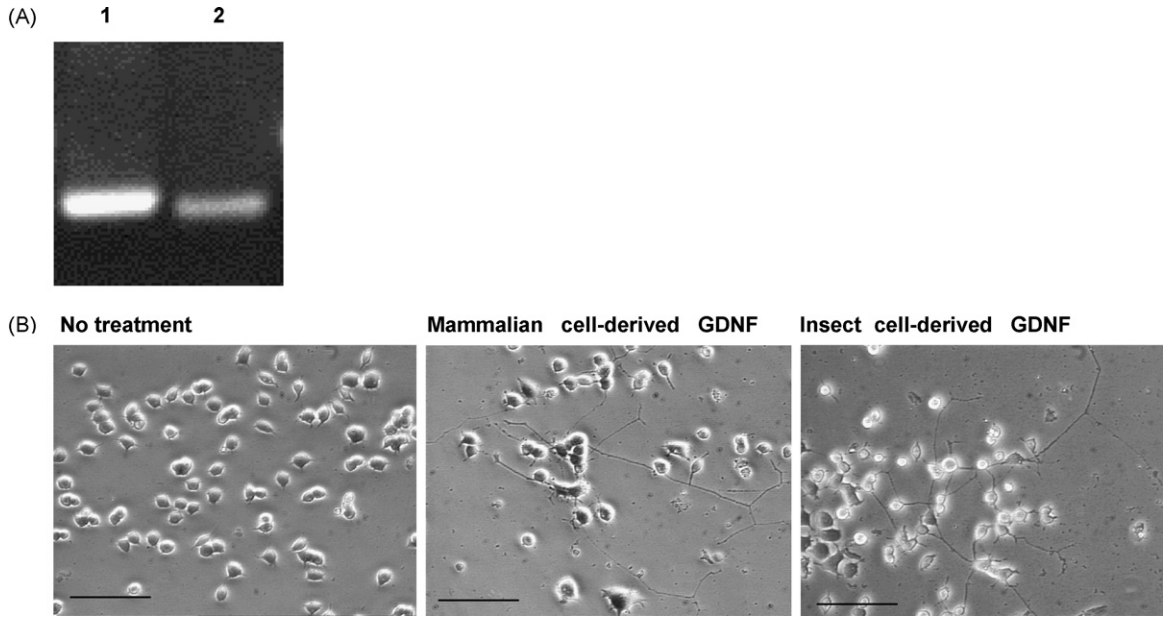

Fig. 6. GDNF-induced differentiation of PC12 cells. (A) The presence of the two GDNF receptors on PC12 was demonstrated by RT-PCR. Lane 1 shows the band for GFRα1 and lane 2 shows the band for RET. (B) PC12 cells were plated on collagen at a low density (2×10^3 cells/cm^2), and treated with 50 ng/ml GDNF on day 0 as indicated. Phase contrast images were taken on day 7. Scale bar 100 μm.

GDNF should be produced in a mammalian expression system. Nonetheless, the use of mammalian expression techniques are time consuming, are difficult to perform on a large scale and yield a low product concentration.

Throughout the purification process, we have used commercially available insect cell-derived GDNF as a positive control because we considered it was structurally closer to the one we were producing. As expected, there were no noticeable differences in biological activity between both proteins. It is well known that N-linked glycans can be dispensable for the function of many proteins although they can also affect conformation, stability or function (Trombetta, 2003). N-Linked glycans account for approximately 25–35% of the molecular mass of GDNF and they seem to be unnecessary for its neurotrophic effect. Bacteria recombinant GDNF infused intraputaminal induced sprouting of dopaminergic fibers in association with clinical improvement in a PD patient (Love et al., 2005) and insect cell-derived GDNF has been described as a neurotrophic factor for peripheral neurons (Trupp et al., 1995). Therefore, further experiments are required to address the role of glycosylation on eukaryotic GDNF. To our knowledge nothing has been done regarding how glycosylation might affect GDNF stability or resistance to proteases. This could be interesting since one of the main issues of GDNF therapy is the need of diffusion of the protein within the parenchyma (Gash et al., 2005). Any strategy that could increase GDNF half-life within the striatum would allow it to reach target cells improving the efficacy of the treatment.

Our final goal is the microencapsulation of GDNF in biodegradable microparticles to evaluate the neuroprotective and regenerative long-term benefits of these systems in vivo in a partial model of Parkinson's disease in rats. We have produced bioactive glycosylated rat GDNF using a mammalian expression system to purify a protein closer to the endogenous GDNF, that might be a more stable and diffusible protein. The same protocol will be used to prepare glycosylated human GDNF for its encapsulation within microparticles that will be tested in the MPTP primate model of PD.

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