Neurosci Bull December 1, 2011, 27(6): 430–435. http://www.neurosci.cn
DOI: 10.1007/s12264-011-1840-8

·Original Article·

Construction and functional activity of a recombinant vector expressing rat glutamic acid decarboxylase 65

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Abstract: Objective Glutamic acid decarboxylase 2 (GAD65) is a gamma-aminobutyric acid (GABA) synthetase. This study aimed to construct a recombinant lentivirus-rGAD65 (rLV-rGAD65) vector containing the cDNA of rat GAD65 (rGAD65) and assess its functional activity *in vitro* and *in vivo*. **Methods** cDNA of rGAD65 was amplified by RT-PCR and subcloned into the LV vector, forming the rLV-GFP-rGAD65 plasmid. The recombinant lentivirus particles (rLV-rGAD65) were packaged by the LV Helper-Free System and the titer was measured. Primary rat lung fibroblasts were transfected with rLV-rGAD65. The expression of rGAD65 in fibroblasts was detected by immunocytochemistry and western blot and the level of GABA in the medium was assessed by high-performance liquid chromatograph (HPLC). *In vivo*, rLV-rGAD65 was injected into the subthalamic nucleus (STN) of Sprague-Dawley rats using stereotaxic methods, and rGAD65 protein levels in the STN were assessed by immunohistochemistry and Western blot, while the GABA concentration in the substantia nigra pars reticulata (SNr) was assayed by HPLC. **Results** The sequence of rGAD65 cDNA was in accord with that in GenBank. The amino-acid sequence of rGAD65 had no mutations and the titer of rLV-rGAD65 reached 6.8×10^8/mL. The efficiency of infection of fibroblasts was 80%, and the concentration of GABA in the medium was (48.14 ± 9.35) nmol/L. *In vivo*, rGAD65 expression was detected in the STN, and the concentration of GABA in the SNr increased from (5.95 ± 1.09) to (12.44 ± 3.79) nmol/g tissue. **Conclusion** The recombinant LV-GFP-rGAD65 vector was successfully constructed. rLV-rGAD65-infected primary fibroblasts *in vitro* and the expressed rGAD65 catalyzed the formation of GABA from glutamic acid. *In vivo*, the concentration of GABA in the SNr was increased after rLV-rGAD65 injection into the STN.

Keywords: rat glutamic acid decarboxylase 2; lentivirus vector; gene clone; Parkinson's disease

1 Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and abnormal motor symptoms such as resting tremor, rigidity and bradykinesia[1-3]. DA neurons have important regulatory functions in the cortex-basal ganglia-thalamus-cortex loop. In PD, these regulatory functions are decreased or lost. The glutamatergic neurons of the subthalamic nucleus (STN) are overexcited and induce an increase in the excitability of gamma-aminobutyric acid

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Article ID: 1673-7067(2011)06-0430-06
Received date: 2011-08-18; Accepted date: 2011-11-08

(GABA) neurons in the substantia nigra pars reticula (SNr) and globus pallidus internus (Gp_i), which are the afferent components of the loop. This may over-inhibit the motor-related nuclei of the thalamus to produce the symptoms of PD. Glutamic acid decarboxylase 2 (GAD65) catalyzes the production of GABA from glutamate, thus changing an excitatory neurotransmitter (glutamate) into an inhibitory one (GABA), decreasing the overexcited state of the Gp_i and SNr. In this manner, GAD65 improves the motor symptoms of PD[4,5]. In the present study, the rat GAD65 (rGAD65) gene was subcloned into the lentivirus (LV) vector, and the functional activity of recombinant LV-rGAD65 (rLV-rGAD65) was assessed *in vivo* and *in vitro*.

2 Materials and methods

2.1 Animals Sprague-Dawley rats (190–210 g) were housed individually and kept under controlled environmental conditions with a 12:12 h light/dark cycle, with light on at 6:00 a.m., for at least one week before the experiments. Standard laboratory chow and tap water were allowed *ad libitum*. All animal experimental procedures were approved by and carried out in accordance with the Medicine Institutional Animal Care and Use Committee of Liaoning Medical University.

2.2 Materials Trizol RNA reagents, diethylpyrocarbonate-treated water and mouse anti-rat antibody targeting GAD65 were all from Sigma (St. Louis, MO, USA) Superscript reverse transcription enzyme was from Invitrogen (Carlsbad, CA, USA). Easy-A high-fidelity PCR cloning enzyme, pGEM-T Easy vector, LV helper-free system (pRsv-REV, pMDlg-pRRE, pMD2G) and lentivirus expression vector LV-GFP-CTB were from Stratagene (La Jolla, CA, USA). The plasmid preparation kit was from Promega (Madison, WI, USA); BamH I, Sal I and DNA markers were from NEB (Beijing, China).

2.3 Primers Based on the sequence of rat GAD65 mRNA (Genbank No. 72422) and the LV-GFP-CTB vector, the upstream primer containing a BamH I site was 5'-CGCGGATCCATGGCATCTCCGGGCTCTGGTC [77–105, coding sequence (CDS): 75–1832], and the downstream primer containing a Sal I site was 5'-CGCACTGAAAGTTTGGTGAGCAAAGTGATTACGA (1823–1857). The primers were synthesized by Shanghai Biotechnology Company, Shanghai.

2.4 rGAD65 cloning

2.4.1 RT-PCR According to the protocol of the kit, total RNA was extracted from the cerebral cortex of one-day-old Sprague-Dawley rats using Trizol RNA reagents. Then cDNA was generated by superscript reverse transcription polymerase, rGAD65 cDNA was amplified by PCR as follows: pre-denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, renaturation at 57°C for 1 min and extension at 72°C for 1 min, followed by extension at 72°C for 5 min. The PCR products were reclaimed with a Glass Milk DNA kit (Beijing Biotechnology Company, Beijing) after 1% agarose gel electrophoresis.

2.4.2 Construction of LV-GFP-rGAD65 The reclaimed PCR products and the pGEM-T easy vector were digested by the BamH I and Sal I restriction endonucleases. The resulting products were purified by 1% agarose gel electrophoresis and reclaimed with the Glass Milk DNA kit. The reclaimed products were connected by T4 DNA ligase at 16°C for 16–20 h. The sequence of *rGAD65* was determined after the connected production was transferred into competent DH5α and the plasmid of pGEM-T-rGAD65 was extracted from it. LV-GFP-CBT and pGEM-T-rGAD65, which contained the correct sequence of *rGAD65*, were digested by BamH I and Sal I. Thus, LV-GFP-rGAD65 was obtained.

2.5 rLV-GFP-rGAD65 packaging and titer determination Based on the protocol of the packaging kit of recombinant lentivirus and the titer-detecting kit of recombinant lentivirus, rLV-GFP-rGAD65 and LV-GFP particles were packaged and their titers were determined. Titer determination was conducted twice.

2.6 rLV-rGAD65 expression *in vitro* Fibroblasts were plated at 3×10^4 cells/well onto a 24-well plate with a glass coverslip in each well. After 24 h, 2×10^5 particles of rLV-rGAD65 or LV-GFP were added to each well in triplicate and incubated for 48 h. Then each well was washed with 200 μL artificial cerebrospinal fluid (CSF) (containing in mmol/L: 144 NaCl, 4 KCl, 1 $MgCl_2$, 5 glucose, and 1.5

$CaCl_2$, pH 7.4) and artificial CSF was added to the 24-well plate (100 μL/well) and incubated for 10 min prior to collection and analysis of GABA by HPLC. Subsequently, 4% paraformaldehyde (4°C, 300 μL/well; Sigma) was added and incubated for 15 min, and the coverslips were rinsed 3 times with PBS (1% Triton X-100, pH 7.4) for 5 min each. The coverslips were stored at 4°C for immunohistochemistry.

The other cells (1×10^6) were digested by trypsinase, harvested, and rinsed 3 times with PBS. Then the proteins were obtained with the total protein extraction reagent (containing proteinase inhibitor, 1:200), and the protein concentration was assessed by the Bradford method. After that, proteins were resolved by SDS-PAGE and transferred to the wet nitrocellulose membrane. After blocking the non-specific protein by bovine serum albumin (BSA), the membranes were incubated with mouse anti-rat GAD65 moloclonal antibody for half an hour at room temperature. Then the membranes were washed with wash buffer for three times and incubated with the alkaline phosphatase-conjugated second antibody (Beijing Biotechnology, Beijing) for 45 min at room temperature. The membranes were washed again and BCIP/NBT (Beijing Biotechnology) was added to visualize the proteins.

2.7 rLV-rGAD65 expression *in vivo* A mixture of 2×10^5 rLV-rGAD65 or LV-GFP and 1 μL manicol was injected into the left STN of normal rats. The rats were sacrificed after 2 weeks. After fixation by intravital perfusion, the rGAD65 expression in the STN region was assessed by immunohistochemistry. Frozen sections through the STN (~40 μm) were immersed in 0.01 mol/L PBST (PBS containing 0.03% Triton-X 100) for 5 min. After the endogenous peroxidase was eliminated by 3% H_2O_2 and the sections were blocked by 5% normal goat serum, they were incubated with mouse anti-rGAD65 antibody at 25°C for 16 h. Then rGAD65 was visualized with DAB.

The SNr and STN were dissected from the unfixed rat brain, ground and prepared for the measurement of GABA content by HPLC (SNr) and rGAD65 content by Western blot (STN) respectively.

2.8 Statistical analysis Data are presented as mean ± SD. Two-sided paired Student's *t*-test was used to compare mean values. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS (version 11.5; SPSS Inc., Chicago, IL, USA).

3 Results

3.1 rGAD65 gene cloning The sequence of the pGEM-T-rGAD65 was very similar to that reported in Genbank (1751/1758; identity rate, 99.60%). The mutated basic groups did not change the amino-acid sequence of the rGAD65 protein.

3.2 Construction of pLV-rGAD65 Plasmids extracted from a random DH5α colony were digested by BamH I and Sal I, resulting in two fragments of 4.8 kb and 1.8 kb on agarose gel electrophoresis (Fig. 1). The 4.8-kb band indicated LV vector and the 1.8-kb band indicated the objective gene–rGAD65.

3. 3 Titer and functional activity of rLV-rGAD65 *in vivo* and *in vitro*

3.3.1 Titer of rLV-rGAD65 The average titer of the constructed rLV-rGAD65 was 6.8×10^8/mL based on testing twice.

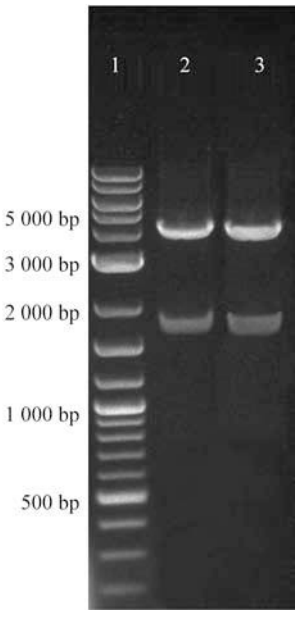

Fig. 1 Electrophoretogram of LV-rGAD65 plasmid digested by BamH I and Sal I. The resulting 4.8-kb band indicated lentivirus vector and the 1.8-kb band indicated the objective gene–rGAD65. Lane 1, marker; lanes 2 and 3, LV-rGAD65.

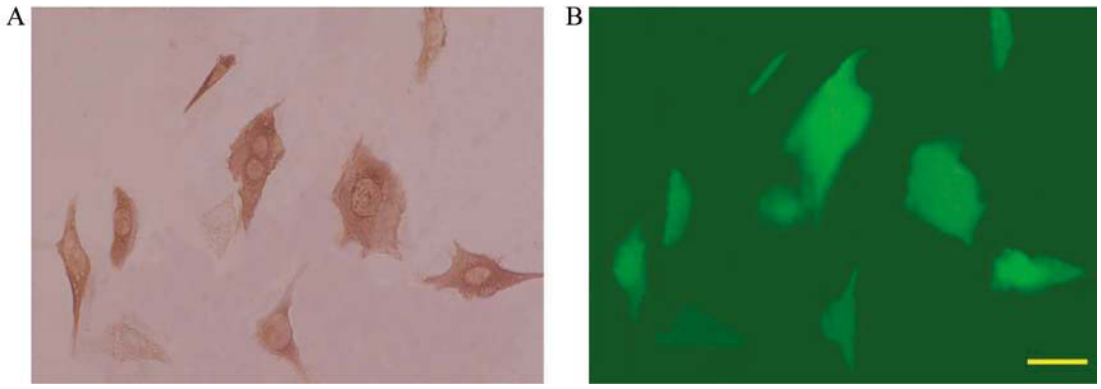

Fig. 2 rGAD65 expression in fibroblasts. A: Immunocytochemical staining of fibroblasts transfected with rLV-rGAD65. The fibroblasts were rGAD65-positive, as indicated by the brown stain. B: The same field as A, under a fluorescence microscope. The fibroblasts showed green fluorescence, confirming that the successfully transfected rLV-rGAD65 was expressed. Scale bar, 20 μm.

3.3.2 Functional activity of rGAD65 *in vitro* Over 80% of fibroblasts transfected with rLV-rGAD65 were rGAD65-positive, as shown by immunocytochemistry (Fig. 2). Furthermore, HPLC revealed that the GABA concentration in the medium was (48.14 ± 9.35) μmol/L (n = 6), while it was almost undetectable in the control group. Moreover, rGAD65 protein was detected by Western blot in the experimental group but not in the control group (Fig. 3)

3.3 Functional activity of rGAD65 *in vivo* Using immunohistochemistry, rGAD65-positive cells were found in the STN ipsilateral to the rLV-GAD65 injection (Fig. 4), while there were almost no positive cells on the opposite

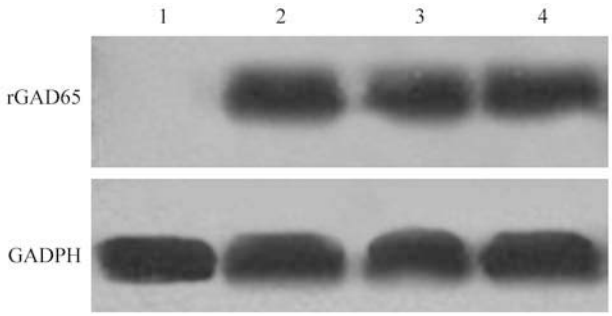

Fig. 3 Western blot of rGAD65 expression in transfected fibroblasts. There was no trace in the control group, but high expression of rGAD65 in fibroblasts transfected with rLV-rGAD65. GAPDH served as the internal control. Lane 1, control fibroblasts without transfection; lanes 2–4, fibroblasts transfected with rLV-rGAD65.

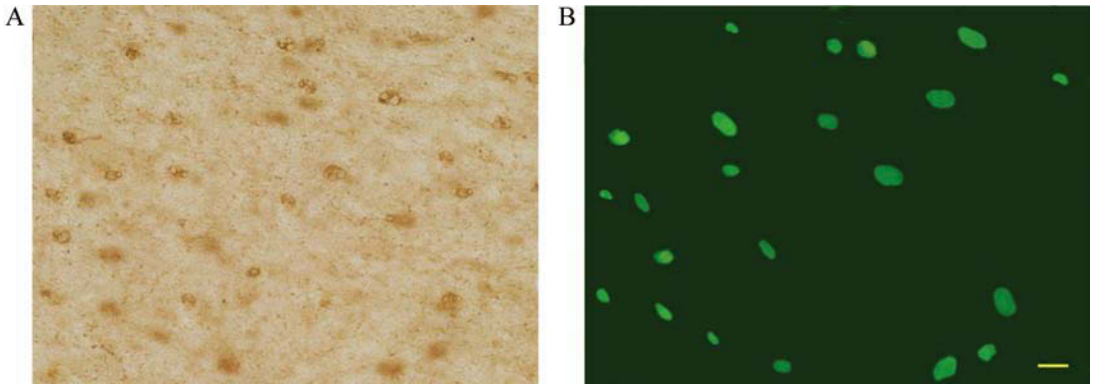

Fig. 4 Expression of rGAD65 in the STN after rLV-rGAD65 injection into the STN. A: Immunohistochemical staining for rGAD65. GAD65-immunoreactive cells were present in the STN ipsilateral to the injection. B: Same visual field as A under a fluorescence microscope. The green blobs show the expression of green fluorescence protein, Scale bar, 20 μm.

side, and on either side in the control group. Consistently, Western blot showed only weak rGAD65 expression in STN on the contralateral side and in the control group, but a high level of rGAD65 protein in STN ipsilateral to the rLV-rGAD65 injection (Fig. 5). Moreover, the GABA content increased from (5.95 ± 1.09) nmol/g on the contralateral side and in the control group (the GABA contents of the control group and in the contralateral side were almost equal, thus merged together) to (12.44 ± 3.79) nmol/g ipsilateral to the injection (P <0.01; n = 5)

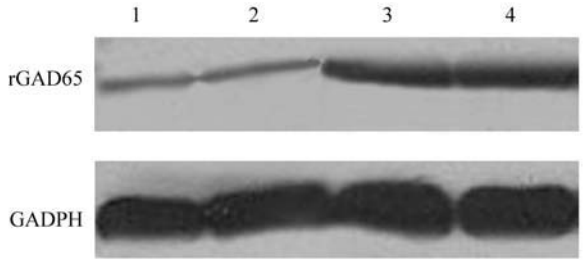

Fig. 5 Western blot of rGAD65 expression in the STN. Results showed only weak rGAD65 expression in the control group (lane 1) and on the contralateral side (lane 2). On the injected side, rGAD65 protein was at a high level (lanes 3 and 4).

4 Discussion

There are two methods of investigating the potential genetic treatment of PD, *in vitro* and *in vivo*, in which the vectors are indispensable[6]. The lentivirus vector has the virulence gene deleted and framework and packaged genes retained. The packaged and framework genes are distributed in different plasmids. Its advantages include large capacity and high transfection efficiency[7]. The titer of the recombinant particles can be as high as 10^8/mL[8]. In our experiment, full-length rGAD65 gene CDS was subcloned into LV-GFP-CBT vector, forming LV-GFP-rGAD65. The plasmids of LV-GFP-rGAD65 and helper system (pRsv-REV, pMDlg-pRRE, pMD2G) were co-transfected into rat lung fibroblasts and cultured for 4 d. After that, the medium was collected and concentrated. The rLV-rGAD65 was harvested and its titer reached 6.8 × 10^9/mL. Moreover, the rLV-rGAD65 construct had a reporter gene, GFP, which can be observed under the fluorescence microscope directly.

GABA is derived from glutamate through decarboxylation catalyzed by GAD, which has two isoforms, GAD65 and GAD67. GAD65 is mainly distributed in the plasma membrane, whereas GAD67 is distributed in the cytoplasm[9]. A previous study by Luo *et al.* showed that GAD65 improves the motor deficit and protects DA neurons in the SNc of a PD rat model, while GAD67 does not[9]. Here, we cloned the rGAD65 gene and constructed the recombinant plasmid LV-GFP-rGAD65. The particles of rLV-rGAD65 were packaged. The effects of rLV-rGAD65 were detected both *in vitro* and *in vivo*, using immunocytochemistry, immunohistochemistry, Western blot and HPLC. The results showed that rGAD65 was cloned successfully, and functioned both *in vitro* and *in vivo*. This suggests that rGAD65 can be used in experiments with the advantage of carrying a reporter gene, which makes it easy to determine the effects of exogenous genes.

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重组大鼠谷氨酸脱羧酶载体的构建和功能分析

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摘要：目的 谷氨酸脱羧酶2 (glutamic acid decarboxylase 2，GAD65) 是γ-氨基丁酸(gamma-aminobutyric acid, GABA)的合成酶。本研究拟构建重组大鼠GAD65基因的慢病毒载体(recombinant lentivirus-rGAD65，rLV-rGAD65)，并在体内外分析其功能。**方法** 用RT-PCR法克隆大鼠GAD65基因的cDNA 并亚克隆至慢病毒载体上，形成重组慢病毒质粒(rLV-GFP-rGAD65)。在包装质粒的帮助下，获得重组慢病毒颗粒(rLV-rGAD65)并检测其滴度。用rLV-rGAD65感染原代培养的大鼠肺成纤维细胞，并用免疫细胞化学和蛋白印迹法检测rGAD65在成纤维细胞中的表达，用高效液相法(high-performance liquid chromatograph, HPLC)检测培养上清中GABA的含量。在体内，rLV-rGAD65 定点注射到Sprague-Dawley大鼠的丘脑底核(subthalamic nucleus，STN)。用免疫组织化学和蛋白印迹法检测GAD65基因在STN中的表达水平，HPLC检测黑质网状部(substantia nigra pars reticulata，SNr) 中GABA的含量。**结果** rGAD65 的序列与GenBank几乎一致，没有碱基的突变。rLV-rGAD65的滴度达6.8×10^8/mL。在体外，rLV-rGAD65对成纤维细胞的感染效率可达80%，而对照组中几乎没有GAD65蛋白的表达。在上清液中，GABA的含量达到了(48.14 ± 9.35) nmol/L。在体内， rGAD65与绿色荧光蛋白共表达于注射侧的STN区，GAD65蛋白的含量明显高于对照组和注射的对侧。GABA在SNr 中的含量也从(5.95 ± 1.09) 升高到(12.44 ± 3.79) nmol/g。**结论** 重组GAD65载体构建成功。在体外，重组慢病毒颗粒可以感染原代培养的成纤维细胞，并能催化GABA的合成。在体内，重组慢病毒颗粒可以感染STN的神经元并能使SNr中GABA的含量增加。

关键词：大鼠谷氨酸脱羧酶2；慢病毒载体；基因克隆；帕金森病