GENERATION OF RECOMBINANT ADENOVIRUS FOR VPS35 EXPRESSION, A PROTEIN IMPLICATED IN PARKINSON'S DISEASE

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Abstract

Introduction: Adenoviral gene transfer is one of the most reliable methods for introducing genes into mammalian cells. Since adenovirus infection is not cell-cycle dependent, it is possible to deliver genes of interest into primary as well as transformed cell lines. VPS35 (Vacuolar Protein Sorting 35) is a subunit of the retromer, a coat-like protein complex involved in the recycling of membrane proteins from endosomes to the trans-Golgi network.Pathological VPS35 mutants are responsible of familial forms of Parkinson's disease (PD). ***Aims:*** Production of recombinant adenovirus for VPS35 expression to study the pathological role of VPS35 in Parkinson's disease. ***Methods:*** Construction of recombinant adenovirus by using Adeno-X Expression System 1, a simple ligation-based system to incorporate a mammalian expression cassette into a replication-incompetent (ΔE1/ΔE3) human adenoviral type 5 (Ad5) genome. ***Results:*** $VPS35^{WT\ or\ D620N}$ recombinant adenoviruses were successfully produced by using Adeno-X Expression System I. Transduction of VPS35 recombinant adenovirus into neuronal cells (SH-SY5Y) determines an high expression level of WT (wild type) or D620N VPS35. ***Conclusion:*** To date, many evidences have indicated the VPS35 involvement in the pathology of neurodegenerative diseases and in particular in PD**.** The $VPS35^{WT\ or\ D620N}$ recombinant adenoviruses will be auseful tool to study the molecular mechanisms by which VPS35 induces neuronal death, and more in general will help to clarify the role of VPS35 in PD.

Key words: VPS35 (Vacuolar Protein Sorting 35), adenovirus vector, genetic transfection.

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1. INTRODUCTION

Parkinson's disease (PD) is a heterogeneous, neurodegenerative disorder affecting 6.3 million people worldwide and 1.2 million in Europe. Mutations in seven genes cause either autosomal dominant (SNCA, LRRK2, VPS35), or autosomal recessive (Parkin, DJ1, PINK1, ATP13A2) familial PD[8].

VPS35 (Vacuolar Protein Sorting 35) is a subunit of the retromer, a coat-like protein complex involved in the recycling membrane proteins from endosomes to the trans-Golgi network. Several lines of evidence have indicated that the VPS35 gene is involved in the pathology of neurodegenerative diseases, including AD (Alzheimer's disease) and PD. Pathogenic mutations of VPS35 may disrupt retromer function in trafficking proteins intracellularly and contribute to dopaminergic neuronal cell death in PD [11]. A single missense mutation, Asp620Asn (D620N), was originally shown to segregate with PD in Swiss and Austrian families, and has been identified in a number of PD subjects and families worldwide [10]. $VPS35^{D620N}$ has been found to act as a loss-of-function mutation perturbing synaptic transmission and affecting neurotransmitter receptor recycling [4]. In a novel viral-mediated gene transfer rat model, the expression of $VPS35^{D620N}$ induces the marked degeneration ofdopaminergic neurons and axonal pathology, a cardinal pathological hallmark of PD [10]. However, the molecular mechanisms by which VPS35 induces neuronal death remains elusive although may share some common pathological pathways with other PD related genes such as LRRK2.

Adenovirus-based vectors have been demonstrated to be excellent gene delivery vehicles in mammals, and certain features of these vectors have made them potential candidates for vaccine development and gene therapy [1]. For example, adenovirus-based vectors can deliver specific genes of interest to a broad spectrum of both actively dividing and post-mitotic quiescent mammalian cells. In addition, the genome of adenovirus vectors

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has large insert capacity and the transgene can be expressed at a high level by strong heterologous promoters. These vectors can also be easily grown to high titers in packaging-cells culture. Moreover, the vector genome rarely integrates into the host chromosome and remains primarily episomal; hence, their use is reasonably safe, with the low risk of insertional mutagenesis [3,14]. The adenovirus type 5 recombinant vectors are the most commonly used system for gene expression by adenovirus. A vector system incorporating deletions in E1 and E3 allow the cloning of about 8.2 kb [5].

In this study, we report for the first time the successful production of VPS35 recombinant adenovirus by using the Adeno-X Expression System 1 (Clontech).Our procedure used conventional in vitro ligation to incorporate VPS35 gene into a replication-incompetent (ΔE1/ΔE3) human adenoviral type 5 (Ad5) genome.

2. MATERIALS AND METHODS

Cell culture

HEK-293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2; SH-SY5Y human neuroblastoma cell line (ATCC number CRL- 2266) were grown in DMEM-F12 (Invitrogen), 10% fetal calf serum (FCS, Invitrogen) at 37°C and 5% CO_2.

Bacteria, plasmid, virus and viral DNA

Wild type and mutant VPS35 cDNA plasmid were kindly provided by Dr. Austen Milerwood (University of British Columbia, Vancouver, Canada); *E. coli* cells (DH5α) were made chemically competent by calcium chloride treatment; pAdeno-X and pShuttle2 vectors(Adeno-X Expression System 1) were purchased by Clontech Laboratories.

Use of restriction and modification enzymes

Restriction enzyme reaction of all DNA were carried out under optimized conditions as described by the providers. The digestion reactions were usually assembled with 3 units enzyme/µg DNA into the specific reaction buffer for 2 hours. For cloning procedure, after the digestion with appropriate enzyme, the vectors were treated, at 37°C, for one hour by Calf Intestinal Phospatase (CIP).

Ligation reaction

Ligase reaction was set up as follows: in a final volume of 15 µl, incubate 100 ng of linearized plasmid and a variable quantity of insert (in a ratio 1:3) were incubated in the presence of 1 unit of T4 DNA ligase (Promega) in an appropriate buffer (Ligase buffer 1X, Promega). After 16 hour of incubation at 16°C, *E. coli* DH5α cells were transformed with ligase reaction.

Transformation of Chemically Competent E. coli DH5α Cells

The chemically competent *E. coli* DH5-α cells were thawed on ice and 100 µl aliquots were incubated with ligase reaction on ice for 30 minutes. The heat shock was performed at 37°C in a water bath for 3 minutes. After the heat shock, the tubes were incubated at room temperature for 10 minutes, then 500 µl LB was added into the tubes and incubated at 37°C for 50 minutes. The cells were plated on an LB plate supplemented with specific antibiotic. The plates were incubated over night at 37°C to grow single colonies.

Transfecting HEK-293 Cells with Pac I-Digested Adeno-X DNA

Approximately 1.5 x 10^6 cells/ml HEK-293 cells were plated in 25 cm^2 flasks 24 hours before transfection. Transient transfection was performed by Lipofectamine LTX Reagent (Life Technologies) according to the manufacturer's instructions using Pac I-digested Adeno-X-VPS35. One week later, the cells were harvested and lysed by three consecutive freeze-thaw cycles. After the third cycle, the cell extracts were briefly centrifuged to remove the pellet debris. Three rounds of virus amplification were performed to obtain high titer of recombinant virus.

Transduction of recombinant adenovirus into SH-SY5Y cells

Neuronal SH-SY5Y cells were transduced in DMEM/F12 medium with scalar concentrations of recombinant adenoviruses encoding wild type or mutant VPS35. 48 hours after transduction, the cells were lysed and analyzed for protein expression by Western blot using ananti-VPS35 antibody (1:1000).

3. RESULTS AND DISCUSSION

Cloning of VPS35$^{WT\ or\ D620N}$ cDNA into pShuttle2

The WT or D620N VPS35 cDNAs were amplified by PCR using specific primers (F-primer-CGAGCG-GCCGCATCCGCCACCATGCCTACAACAC and R-primer- GTCGGTACCTTAGGTACGCGCGTAGAATCGAGAC-CGAG) and a plasmid template kindly provided by Dr. Austen Milerwood (University of British Columbia, Vancouver, Canada). The amplification steps involved initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, polymerization at 72°C for 5 minutes and final polymerization at

72°C for 10 min. The PCR products (2400 bp) and pShuttle2 (3939 bp) vector were digested by KpnI and NotI restriction enzymes (Figure 1A).The VPS35 cDNAs and the linearized pShuttle2 digestion products were purified from agarose gel by Wizard® SV Gel and PCR Clean-Up System kit (Promega).Then digested products were ligated and tranformed into the DH5α *E.coli*. Selection of colonies to identifyre-combinant pSh-VPS35$^{WT/D620N}$ was performed by digestion of the purified plasmid by EcoRI.

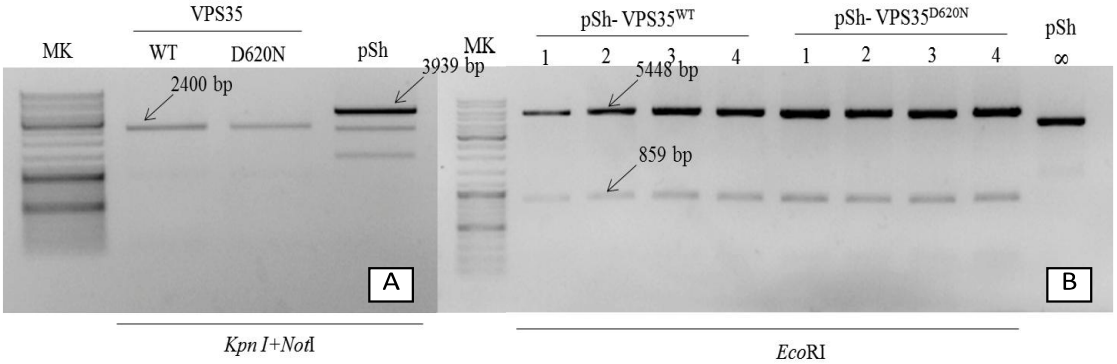

Figure 1. A.Digestion of the pShuttle2 vector and PCR products by *KpnI* and *NotI*. . Lane 1: Molecular marker; lane 2: digestion of VPS35^{WT}; lane 3: digestion of VPS35^{D620N}; lane 4: digestion of pShuttle2; **B.Control the positive clones after ligase reaction by digestion with *Eco*RI.** Lane 1: Molecular marker; lane 2-5: digested pSh-VPS35^{WT} samples; lane 6-9: digested pSh-VPS35^{D620N} samples; lane 10: supercoiled pShuttle2.

The two expected bands (5448 bp and 859 bp) of pSh-VPS35$^{WT\ or\ D620N}$ were clearly visible on agarose gel from lane 2 to lane 9 (Figure 1B).

Production of recombinant adenoviral DNA containing VPS35$^{WT\ or\ D620N}$ cDNAs

Expression cassette containing VPS35 was excised from pShuttle2 (by digestion with*Cla*I, *I-Ceu*I, and *PI-Sce*I) (Figure 2) and ligated into Adeno-X Viral DNA and tranformed into DH5α E.coli. Single colonies were selected and the pAdnoX-VPS35$^{WT\ or\ D620N}$plasmid were identified by XhoI digestion (Figure 3).

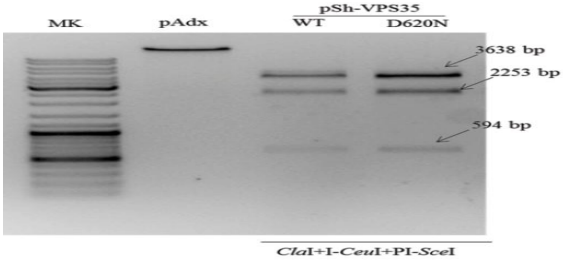

Figure 2. *Cla*I; I-*Ceu*I and PI-SceI digestion of pShuttle2-VPS35$^{WT/D620N}$ and pAdeno-X. Lane 1: Molecular marker; lane 2: digested pAdenoX vector; lane 3: digested pSh-VPS35^{WT}; lane 4: digested pSh-VPS35^{D620N}.

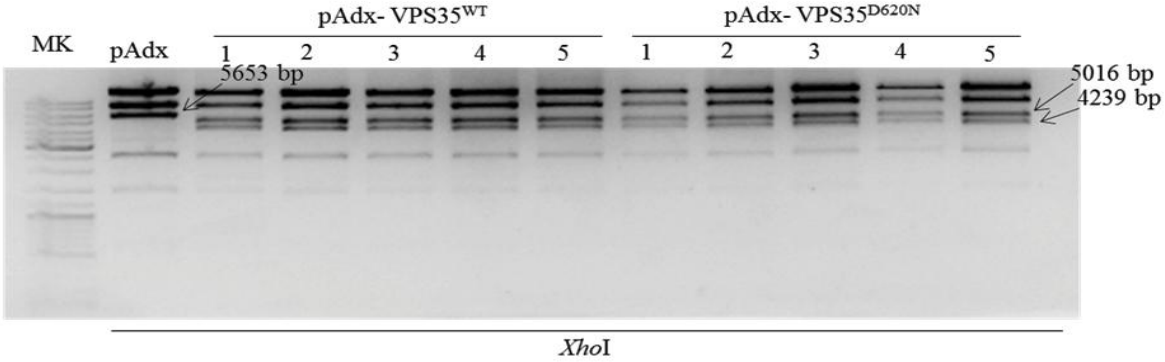

Figure 3. Control of recombinant Adenovirus encoding VPS35$^{WT\ or\ D620N}$. Lane 1: Molecular marker; lane 2: pAdenoX vector. lane 3-7: digested pAdx-VPS35^{WT} samples; lane 8-12: digested pAdx-VPS35 ^{D620N} samples;

Transfection of recombinant adenoviral vector containing VPS35$^{WT\ or\ D620N}$ into HEK-293 packaging cells.

For the production of Adeno-X VPS35$^{WT\ or\ D620N}$ viral particles, the recombinant plasmids were digested by *Pac*I restriction enzyme to expose the inverted terminal repeats (ITRs) located at the ends of the genome. Adeno-X VPS35$^{WT\ or\ D620N}$ digested by *Pac*I produced three visible bands (33334 bp, 2963

bp, 10 bp) on the agarose gel, that are showed in Figure 4. DNA fragment of 33334 bp containing VPS35$^{WT\ or\ D620N}$ expression cassette was purified on agarose gel and used for transfection.

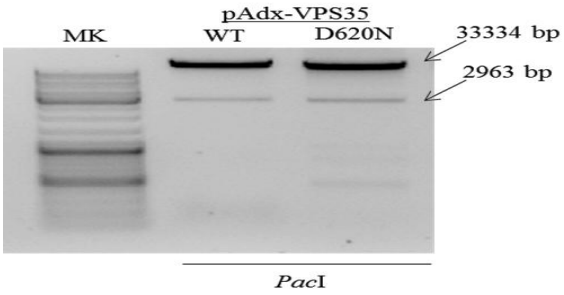

Figure 4. *Pac*I digestion of pAdenoX-VPS35 $^{WT\ or\ D620N}$. Lane 1: Molecular marker; lane 2: digested product of pAdx-VPS35 WT, lane 3: digested product of pAdx-VPS35.

Transfection of recombinant adenoviral vector containing VPS35$^{WT\ or\ D620N}$ into HEK-293 packaging cells was performed by liposomes. After three rounds of virus amplification, high titer of recombinant virus was obtained.

Finally, neuronal SH-SY5Y cells were transduced with scalar concentrations of recombinant adenoviruses encoding wild type or mutant VPS35. The expression levels of all proteins were analyzed by immunoblotting using a specific anti-VPS35 antibody (Figure 5).

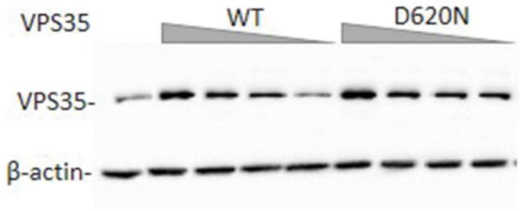

Figure 5. Analysis of the VPS35 expression on SH-SY5Y cells were transduced

The transduction determines a significant increase of VPS35 compared to basal level (Figure 5 lane 1) and this increase correlates with the amount of adenovirus titer.

Pathogenic mutations of VPS35 may disrupt retromer function in trafficking proteins in neuronal cells contributing to dopaminergic neuronal cell death in PD. Decreased VPS35 levels in Drosophila dopaminergic neurons were shown to lead to locomotor defects and reduced life span. In addition, ectopic expression of VPS35^{D620N} in the substantia nigra of rat brains was shown to cause a loss of dopaminergic neurons[12]. Zavodszky et al. found that VPS35^{D620N} mutation destabilizes the retromer interation with WASH (Wiskott–Aldrich syndrome protein and SCAR homolog) complex, resulting in reduced endosomal localization of the WASH complex and consequently in significant defects in endosomal/lysosomal trafficking [13]. A recent study has shown that the VPS35 D620N mutation disrupts the trafficking of cathepsin D, a protein responsible for the degradation of α-synuclein. Interestingly, cathepsin D knockout mice show increased misfolding and cellular toxicity of α-synuclein in dopaminergic neurons[2; 3; 6].Moreover, VPS35 and retromer are involved in endosomal recycling of GluR1 AMPAR subunits. The D620N mutation affects the ability of VPS35 to properly traffick these receptor subunits and produces significant alterations in synaptic transmission [7]. Moreover different experimental evidence suggest a potential role of VPS35 expression in mitochondrial fusion and function, an event critical for DA neuron survival [9].

In our study, we were able to produce VPS35$^{WT\ or\ D620N}$ recombinant adenoviruses using Adeno-X Expression System I. The VPS35$^{WT\ or\ D620N}$ recombinant adenoviruses will be an useful tool to study the molecular mechanisms by which VPS35 induces neuronal deathin Parkinson's disease.

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