Journal of Biotechnology 284 (2018) 1–5

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Short communication

Molecular biology tools for the study and therapy of PDE6β mutations

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ARTICLE INFO

Keywords:
PDE6β
Expression systems
Retinal pigment epithelium
Protein expression

ABSTRACT

Gene therapy has the potential for treating retinal diseases, and we have been developing delivery vehicles and expression vectors for this purpose. In this short communication, we describe the generation of tools for both *in vitro* studies of the disease mechanism and for *in vivo* testing of therapeutic approaches. We have cloned the PDE6β gene and also recreated the same mutation present in the rd10 mouse using an optimized plasmid vector. To allow visual detection, we have also generated, through site-directed mutagenesis, plasmids expressing the normal and mutated PDE6β gene fused with the GFP gene. Next, we have transfected retinal pigment epithelium cells with the different vectors and detected the protein expression of both the normal and mutated PDE6β. With this work we have created gene therapy tools for *in vitro* and *in vivo* studies of retinal disease-causing mutations, namely for the PDE6β, implicated in retinitis pigmentosa.

1. Introduction

Gene therapy has already been shown to have therapeutic efficiency in treating retinal diseases such as Lebers's Congenital Amaurosis (Pierce and Bennett, 2015; Bennett et al., 2016; Le Meur et al., 2018), choroideremia (MacLaren et al., 2014; Barnard et al., 2014), and others (Grob et al., 2016). For gene therapy to be successful, there are three aspects that need to be taken into account: 1) the delivery vehicle, 2) the gene expression system, and 3) the knowledge of the disease mechanism, for tailoring the therapy. In this work, we aimed to generate adequate gene expression systems that would simultaneously allow studying the disease mechanism *in vitro* and to test the therapeutic potential *in vivo* in adequate mouse models of retinal diseases. Therefore, we have chosen to model Phosphodiesterase 6 (PDE6), an important enzyme of the visual cycle, as it is responsible for the hydrolysis of cyclic guanine monophosphate (cGMP) to GMP, necessary for the normal function of photoreceptor cells during the visual cycle. PDE6 in rod photoreceptors consists of two catalytic subunits - PDE6α and PDE6β - and two regulatory/ inhibitory PDE6γ subunits. Diseases associated with mutations in the Rod cGMP-Specific 3,5-Cyclic PDE6β subunit represent about 4–5% of all Retinitis Pigmentosa cases (Ferrari et al., 2011; Hartong et al., 2006).

There are two mouse models with spontaneous mutations in the PDE6β: the rd1 and rd10. The first model (rd1) has a deletion in the PDE6β gene which leads to severe degeneration of photoreceptors starting at postnatal day 8 (P8), progressing to complete rod loss by P20. The second model, rd10, has a missense mutation in the PDE6β sequence (gene position 1678, protein position 560) with an autosomal recessive inheritance, with rods beginning to degenerate from the center to the periphery of the retina between P16 and P20, reaching maximum cell death between P21 and P25. Both models present significant functional alterations, detected by electroretinography (ERG) changes in a-waves (cone and rods photoreceptors) and b-waves (Muller and ON-bipolar cells) (Barhoum et al., 2008; Pennesi et al., 2012). However, the rd10 model shows a more accurate simulation of the disease process in humans than the rd1 mouse since the phenotype starts approximately 8 days later, which allows a larger therapeutic window (Pennesi et al., 2012), being therefore more suited for studying therapeutic approaches, such as those based on gene therapy. What is yet unclear is the mechanism by which the PDE6β mutation found in rd10 contributes to the disease onset. Therefore, to create an *in vitro* model to study the PDE6β mutation, we have recreated the mutation present in rd10 mice using an optimized expression vector (pEPito), which we have previously used for sustained gene expression in the retina (Calado et al., 2016a, 2014). Simultaneously, we have cloned the normal mouse PDE6β into pEPito for assessment of the therapeutic potential in the rd 10 mouse model bearing this mutation. With these expression vectors, we have evaluated the expression of the normal and

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https://doi.org/10.1016/j.jbiotec.2018.07.030
Received 7 May 2018; Accepted 24 July 2018
Available online 24 July 2018
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mutated protein in an *in vitro* system using retinal pigment epithelium cells, and we here show that it is possible to recreate the mutation *in vitro* and to express and detect the normal PDE6β in the rd10 mouse.

2. Materials and methods

2.1. Cell line

A human retinal pigment epithelium cell line (D407) was used for the *in vitro* experiments (Calado et al., 2016b; Simao et al., 2016; Bitoque et al., 2017; Machado et al., 2014; Davis et al., 1995). Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich), and 5% fetal bovine serum (FBS, Sigma-Aldrich). Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

2.2. Plasmid construction

pEPito-hCMVeGFP plasmid containing the human CMV enhancer/human elongation factor 1 alpha promoter was used as backbone (Haase et al., 2010). Mouse PDE6β was amplified from the pAd.CMVβPDE plasmid (9.9 kb), with specific primers:

Forward: 5′ GATCGCTAGCAATGAGCCTCAGTGAGGAA 3′

Reverse: 5′ GATCACCGGTTTATAGGATACAGCAGGTCG 3′

with NheI and AgeI restriction sites, respectively. The amplified fragment was digested with NheI and AgeI and cloned into pEPito-hCMV, previously digested with the same enzymes. The construct pEPito-hCMV-PDE6β, with 7814bp, was confirmed by restriction enzymes and Sanger sequencing. The plasmid was further grown in *Escherichia coli* GT115 competent cells, extracted and purified using Qiagen's Mini-Prep kit according to the manufacturer's instructions.

2.3. Mutagenesis of pEPito-hCMV-PDE6β plasmid

To mutate the PDE6β gene we have performed a site-directed mutagenesis by designing specific primers using the PrimerX web-based tool and the Pfu-Turbo enzyme (Agilent) to introduce a missense mutation in the gene position 1678 c > t (Primer pair 1), to generate the pEPito-hCMV-PDE6β^{R560C} plasmid (Supplementary data 1). The STOP codon at the end of the PDE6β gene of the pEPito-hCMV-PDE6β and pEPito-hCMV-PDE6β^{R560C} plasmids was eliminated by the deletion of one nucleotide (t), which allowed the GFP gene to become in-frame (Primer pair 2), originating plasmids pEPito-hCMVPDE6βeGFP and pEPito-hCMV-PDE6β ^{R560C}eGFP.

```
Primer                         pair                              1
                                 *
Forward:   5'   CCTACCACAACTGGTGCCACGGCTTCAATG        3'
Reverse:   5'   CATTGAAGCCGTGGCACCAGTTGTGGTAGG        3'
                                   *
Primer pair 2
                             \ /
Forward: 5' CTGCTGTATCCTAAAACCGGTCGCC 3'
Reverse: 5' GGCGACCGGTTTTAGGATACAGCAG 3'
                             /                                  \
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The reaction mixture was prepared with 20 ng of plasmid, 1 µL each primer (10µM), 0.5 µL DMSO, 0.5 µL Pfu-Turbo enzyme (2.5U/µL), polymerase buffer and Molecular grade H_2O for a final volume of 25 µL. Eighteen amplification cycles were performed in a C1000™ Thermal Cycler (Biorad) with the following setup: an initial denaturation step for 1 min at 95 °C, a denaturation step with a temperature of 95 °C for 50 seg, an annealing step with a temperature of 60 °C for 1 min, an extension step with a temperature of 68 °C for 8 min and a final extension step for 10 min at 68 °C. After amplification, the product was digested overnight with 0.5 µL of DpnI (Promega) that digests CpG sites that were methylated by bacteria thus eliminating plasmids without the mutation. We have next transformed and grown batches of *E. coli* GT115 competent cells (Invitrogen) with each of the plasmids, which were extracted and purified as described before. We have further confirmed the mutations by Sanger sequencing. CLUSTAL Omega multiple sequence alignment program was used to align and confirm the mutagenesis (Sievers and Higgins, 2014a, b).

2.4. In vitro transfection and expression analysis

To assess if the constructed plasmid was expressing the PDE6β protein, 4×10^4 cells were seeded in glass coverslips (13 mm of diameter) in a 24-well tissue culture plate (Orange Scientific). 24 h after seeding, transfection was performed using FuGENE® HD (Promega) with a 3:1 (µL of reagent: µg of DNA) ratio, according to the manufacturer's instructions. After 48 h, the cells were fixed with methanol, immunoassayed for PDE6β (1/500, ThermoFisher PA1-722) and visualized in a wide field fluorescence microscope (Zeiss Z2).

For Western blot evaluation of PDE6β expression, a higher number of cells (1×10^5 cells) were seeded in glass coverslips in a 6-well tissue culture plate (Orange Scientific) to obtain an appropriate protein concentration and quantity. After 24 h, the cells were transfected with the different plasmids using FuGENE® HD (Promega) with a 3:1 (µL of reagent: µg of DNA) ratio, according to the manufacturer's instructions. After 72 h, the culture medium was discarded, cells were washed with PBS 1X and retrieved with 100 µL of ice-cold Radio-Immunoprecipitation Assay (RIPA) lysis buffer, supplemented with a protease and phosphatase inhibitor cocktail (Roche and Sigma-Aldrich) and incubated on ice for 20 min. After centrifugation (16 200 g for 20 min at 4 °C), the supernatant was collected and protein concentration was determined using the Braford protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as standard. Protein samples were mixed with 4x SDS (sodium dodecyl sulphate) sample buffer, heated for 5 min at 95 °C and 30 µg of protein were electrophoresed at 100 V for 2 h in a 10% SDS. After proteins were electrotransferred (at 20 V for 20 min) onto polyvinylidinedifluoride (PVDF) membranes, the Transblot sheets were blocked with 5% BSA (in TBS-T (0.1%Tween-20) for 2 h at room temperature. The membrane was incubated overnight at 4 °C with PDE6β antibody (Santa Cruz Biotechnology, sc-377486) at a dilution of 1/500, with 5% BSA in TBS-T. To ensure that samples were evenly loaded, the membranes were re-probed for β-actin (1/3000; Santa Cruz Biotechnology) for 1 h. The membrane was subsequently washed with TBS-T and then incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase, 1/5000 (Santa Cruz Biotechnology) for 1 h at room temperature. Afterwards the membranes were washed with TBS-T for 20 min and TBS for 5 min. After incubation with the enhanced chemiluminescence detection reagent (ECL, GE Healthcare) for 5 min, the membranes were visualized in a ChemiDoc Touch (Bio-Rad).

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