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Cell models for McArdle disease and aminoglycoside-induced read-through of a premature termination codon

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Abstract

McArdle disease results from mutations in the gene encoding muscle glycogen phosphorylase (PYGM) protein and the two most common mutations are a premature termination codon (R50X) and a missense mutation (G205S). Myoblasts from patients cannot be used to create a cell model of McArdle disease because even normal myoblasts produce little or no PYGM protein in cell culture. We therefore created cell models by expressing wild-type or mutant (R50X or G205S) PYGM from cDNA integrated into the genome of Chinese hamster ovary cells. These cell lines enable the study of McArdle mutations in the absence of nonsense-mediated decay of mRNA transcripts. Although all cell lines produced stable mRNA, only wild-type produced detectable PYGM protein. Our data suggest that the G205S mutation affects PYGM by causing misfolding and accelerated protein turnover. Using the N-terminal region of PYGM containing the R50X mutation fused to green fluorescent protein, we were able to demonstrate both small amounts of truncated protein production and read-through of the R50X premature termination codon induced by the aminoglycoside, G418.

Keywords: Glycogen phosphorylase; PYGM; McArdle disease; Glycogen storage disease V; Aminoglycoside read-through; Premature termination codon; Cell model

1. Introduction

McArdle disease (glycogen storage disease V) is caused by a defect in glycogenolysis within skeletal muscle cells [1]. It results in an inability to utilise glycogen as an energy source for anaerobic muscle contraction and aerobic glycolysis. Affected people complain of exercise intolerance with fatigue and myalgia and are at risk of acute episodes of rhabdomyolysis [2]. McArdle disease is caused by mutations in the *PYGM* gene that encodes the muscle glycogen phosphorylase enzyme. To date, over 150 causative mutations in the *PYGM* gene have been identified. The R50X premature termination codon is the most common mutation and the G205S missense mutation is the second most common mutation in Caucasians in Europe and North America. Allele frequencies vary between countries, with recent reports of allele frequencies of 54–77% for R50X and of 3–10% for G205S [3,4]. The R50X mutation results in an absence of mRNA due to nonsense-mediated decay [5–7]. The G205S mutation is located within a domain involved in glycogen binding and tetramerisation [8]. It results in stable mRNA, but the mutation prevents PYGM enzymatic function [9–11], although the exact mechanism of this is not known.

At present there is no effective treatment for the disorder, although regular aerobic exercise is not harmful and may be beneficial through aerobic conditioning [3]. Enzyme replacement therapy is a successful treatment

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for some lysosomal storage diseases, including Type 1 Gaucher disease and Pompe disease (glycogen storage disease II) [12,13]. However, enzyme replacement therapy is not a suitable treatment for McArdle disease as wild-type PYGM protein is cytoplasmic and the replacement enzyme would be directed to the lysosome rather than the cytoplasm. If nonsense-mediated decay could be suppressed, then read-through of the R50X premature termination codon in order to produce full-length PYGM protein would be a potential treatment strategy for McArdle disease patients with this mutant allele providing the resultant protein was enzymatically active. Aminoglycoside antibiotics such as gentamicin, or novel non-aminoglycoside compounds (e.g. Ataluren/PTC124) have been shown to induce read-through of premature termination codons in clinical trials in cystic fibrosis and Duchenne muscular dystrophy patients [14–16]. Some aminoglycoside read-through drugs may have the dual effect of suppressing nonsense-mediated decay and inducing read-through of the premature termination codon [17–19]. The exact mechanism of how the G205S mutation disrupts PYGM enzyme activity is not known, but elucidating this could suggest potential treatments for patients with this mutation.

Cell models play a valuable role in improving understanding of the molecular mechanisms of disease and for testing treatments. A model of the R50X and G205S mutations is required to test and identify potential therapies for McArdle disease. At present there are ovine and bovine models of the disease [20–22], but neither has the R50X or G205S mutations. For many muscle diseases, cultured muscle cells from patients can be used to produce an *in vitro* cell model. However, this is not possible for McArdle disease because, although PYGM protein is expressed at high levels in skeletal muscle *in vivo*, cultured wild-type muscle cells do not mature sufficiently and only express the protein at very low levels [23].

Here we describe the creation of cell models expressing wild-type PYGM, or the two most common mutants (R50X and G205S), from cDNA. To overcome problems of low-level PYGM expression from muscle cells in culture, our cell models were created by transient and stable transfection with cDNA under the control of a human cytomegalovirus (CMV) promoter for high-level constitutive expression. We show that cloned cell lines with genome-integrated cDNA produced stable mRNA from all three cDNAs, since nonsense-mediated decay cannot operate on intronless RNA transcripts. We demonstrate aminoglycoside-induced read-though of a construct containing the R50X premature termination mutation using G418. We also provide experimental evidence that misfolding and aggregation of mutant PYGM may partly explain why the G205S mutation causes McArdle disease.

2. Materials and methods

2.1. Plasmid constructs

pCMV-SPORT6 plasmid containing mouse *Pygm* cDNA (NCBI Accession BC012961) was obtained from the I.M.A.G.E. consortium (I.M.A.G.E. ID 3989941) [24,25]. Mouse *Pygm* cDNA was used because full-length human *PYGM* cDNA was not available when these studies were performed, and mouse and human PYGM amino acid sequences have high amino acid identity (97%). The cDNA was transferred to pCI-neo (Promega, Madison, WI) using compatible SalI and NotI restriction sites. The QuikChange XL site-directed mutagenesis kit (Stratagene-Agilent, Santa Clara, CA) was used to introduce the specific base changes. The R50X mutation was introduced into *Pygm* using forward primer 5′-GTG GCT ACT CCG TGA GAT TAC TAT TTT GC-3′ and reverse primer 5′-GCA AAA TAG TAA TCT CTC GGA GTA GCC ACA TTG CG-3′ to change AGA to TGA. The G205S mutation was introduced into *Pygm* using forward primer 5′-CTG CCT GTG CAT TTC TAT AGC CGA GTG GAG C-3′ and reverse primer 5′-GCT CCA CTC GGC TAT AGA AAT GCA CAG GCA G-3′ to change GGC to AGC.

To introduce N-terminal GFP-encoding sequences (Fig. 1), *GFP* cDNA was excised from pEGFP-C1 (Clontech, Mountain View, CA) and cloned into pCI-neo using NheI and XhoI restriction sites on both plasmids. Wild-type and mutant *Pygm* were then cloned from pCI-neo into the *GFP*-pCI-neo constructs with MluI and NotI.

For read-through studies, constructs encoding C-terminal fusions of GFP to the 50 amino acids of wild-type (R50R) or 49 amino acids of mutant (R50X) PYGM protein were produced (Fig. 1). PCR was performed using a common forward primer (5′-CGC TGC TAG CGC CAT GTC CAG GCC TCT TTC-3′) and either a reverse primer encoding a NheI site (5′-CGG GGC TAG CTC TCT CGG AGT AGC CAC ATT-3′) on the wild-type template, or a reverse primer encoding a NheI site and stop codon (5′-CGG GGC TAG CTC TCA CGG AGT AGC CAC ATT-3′) on the R50X *Pygm* template in pCI-neo. The PCR products were digested with NheI and cloned into the NheI site of pCI-neo. Plasmids from positive colonies were sequenced to confirm orientation, reading-frame and single insertion. To remove the neomycin cassette from these R50X-GFP and R50R-GFP constructs, they were digested with AvrII and BstBI, blunt-ended and religated (Fig. 1).

Predicted sizes of recombinant proteins are: (a) GFP-PYGM and GFP-G205S: GFP + 20aa linker + PYGM = 127 kDa, (b) GFP only: GFP + 36aa linker to first pCI-neo stop codon = 31 kDa, (c) GFP-R50X: GFP + 20aa linker + 6 kDa (49aa) = 35 kDa, (d) R50R-GFP: 50aa of PYGM + 8aa linker + GFP + 36aa linker to first pCI-neo stop codon = 37.5 kDa.