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A highly prevalent equine glycogen storage disease is explained by constitutive activation of a mutant glycogen synthase

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ABSTRACT

Background: Equine type 1 polysaccharide storage myopathy (PSSM1) is associated with a missense mutation (R309H) in the glycogen synthase (*GYS1*) gene, enhanced glycogen synthase (GS) activity and excessive glycogen and amylopectate inclusions in muscle.
Methods: Equine muscle biochemical and recombinant enzyme kinetic assays in vitro and homology modelling in silico, were used to investigate the hypothesis that higher GS activity in affected horse muscle is caused by higher GS expression, dysregulation, or constitutive activation via a conformational change.
Results: PSSM1-affected horse muscle had significantly higher glycogen content than control horse muscle despite no difference in GS expression. GS activity was significantly higher in muscle from homozygous mutants than from heterozygote and control horses, in the absence and presence of the allosteric regulator, glucose 6 phosphate (G6P). Muscle from homozygous mutant horses also had significantly increased GS phosphorylation at sites 2 + 2a and significantly higher AMPKα1 (an upstream kinase) expression than controls, likely reflecting a physiological attempt to reduce GS enzyme activity. Recombinant mutant GS was highly active with a considerably lower K_m for UDP-glucose, in the presence and absence of G6P, when compared to wild type GS, and despite its phosphorylation.
Conclusions: Elevated activity of the mutant enzyme is associated with ineffective regulation via phosphorylation rendering it constitutively active. Modelling suggested that the mutation disrupts a salt bridge that normally stabilises the basal state, shifting the equilibrium to the enzyme's active state.
General significance: This study explains the gain of function pathogenesis in this highly prevalent polyglucosan myopathy.

1. Introduction

The ability to store glucose as the branched-chain polymer glycogen is shared by organisms as evolutionarily diverse as bacteria and mammals: it enables organisms to deal with temporary starvation by maintaining energy provision through glycogen catabolism, and, in times of plenty, to store this energy macromolecule substrate in a form that has minimal effect on cellular osmotic pressure [1]. In mammals, glycogen is normally stored in muscle and other tissues following the action of 2 key enzymes: glycogen synthase (GS) and glycogen branching enzyme (GBE). GS catalyses the polymerisation of glucose from a UDP-glucose (UDPG) substrate via α1–4 glycosidic bonds and GBE is responsible for generating α1–6 bonds, every 4–10 residues [2]. There are 2 GS isoforms: one, encoded by the *GYS1* gene and predominantly expressed in muscle but also in other tissues and the other encoded by the *GYS2* gene and expressed only in liver. Both GS isoforms are regulated by protein kinase-catalysed phosphorylation and allosteric modulation by glucose-6-phosphate (G6P) [3].

There are more than 14 glycogen storage diseases in humans and animals [4–7], several of which are characterised by excessive muscle glycogen or polyglucosan and caused by enzymatic defects in glycogenesis or glycogenolysis. In 2008, a missense, autosomal dominant mutation (R309H) in *GYS1* was identified in Quarter Horses with polysaccharide

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storage myopathy (PSSM1) [8], a disease characterised by intermittent exertional rhabdomyolysis episodes and excessive muscle glycogen and amylopectate accumulation [9,10] but with an absence of cardiac signs [11]. The R309H mutation is associated with increased GS activity in muscle from affected horses [8]. The identical mutation has now been reported in many horse breeds worldwide [12–15] suggesting dissemination from a single founder and in certain breeds, positive selection [16] perhaps due to the increased muscle glycogen storage in affected animals [8]. Since the ratio of GS:GBE activity is important in the formation of glycogen with a normal structure [17], filamentous alpha crystalline polysaccharide that forms in PSSM1-affected muscle likely develops because of an increased GS:GBE activity ratio [18] as seen in transgenic mice with constitutively active muscle GS [19]. The mechanism by which the equine R309H mutation results in increased GS activity is unknown; potential mechanisms include elevated GS expression, reduced degradation or dysregulation of enzyme activity. However, since the R309H mutation occurs in a highly conserved region of all GS enzymes and a mutation of an adjacent amino acid in yeast GS (equivalent to amino acid G310 in mammalian GS) also results in increased enzyme activity [20], it is possible that mutations in this region cause constitutive enzyme activation.

Covalent regulation of GS is dependent on 9 separate phosphorylation sites that are modified by various upstream kinases; of these, phosphorylation at sites 2 (serine residue S7), 2a (S10), 3a (S641) and 3b (S645) decrease the enzyme activity more than the remaining 5 sites [21–23]. Furthermore, phosphorylation of certain sites enables sequential phosphorylation of others so that they can be functionally grouped for investigative purposes [24]. Two of the key upstream kinases responsible for marked inhibition of GS are glycogen synthase kinase 3β (GSK3β) (which phosphorylates sites 3a, 3b, 3c, and 3d) and AMP-activated protein kinase (AMPK), which phosphorylates GS at site 2. Other kinases, namely Casein Kinase 2 and Protein kinase A, are associated with phosphorylation at sites 1a, 1b and 5 which have little effect on GS activity [24]. The phosphorylation of GS has a significant effect on G6P affinity; indeed, regulation by G6P is associated with a feed-forward mechanism that causes further enzyme activation by dephosphorylation [25]. Although the R309H residue is distant in the primary sequence from any of the known phosphorylation sites, the mutation could result in an inappropriate response to phosphorylation as is seen in a yeast GS variant [20].

Although the crystal structure has not been published for any mammalian GS, the structures for a bacterial GT5 synthase (*Agrobacterium tumefaciens*) [2] and, more recently, for a yeast synthase (*Saccharomyces cerevisiae*; Gsy2p) [26] and a *Caenorhabditis elegans* synthase [27] have been solved. Yeast GS is a homo-tetrameric protein in both its basal and active states and eukaryotic GT3 synthases likely have a similar tetrameric arrangement due to their high sequence identity [26]. Since the amino acid sequence surrounding the PSSM1 R309H *GYS1* mutation is highly conserved amongst species [8] it is possible that this site might have an important (but as yet unknown) functional role [2,28]. It is not however thought to be involved in substrate binding [2,29] or catalytic activity [2,20,30].

The binding of the G6P ligand to GS is complex [26]. The structural basis for G6P allosteric activation has been revealed from analysis of high-resolution crystal structures of the homologous synthase enzyme, Gsy2p from *Saccharomyces cerevisiae*: GS has a binding pocket for the phosphate side chain of G6P that comprises 5 amino acid residues that are conserved across the GT3 family glycosyltransferases [26]. G6P binding results in a major conformational change involving translation and rotation toward the tetramer interface of the enzyme to generate

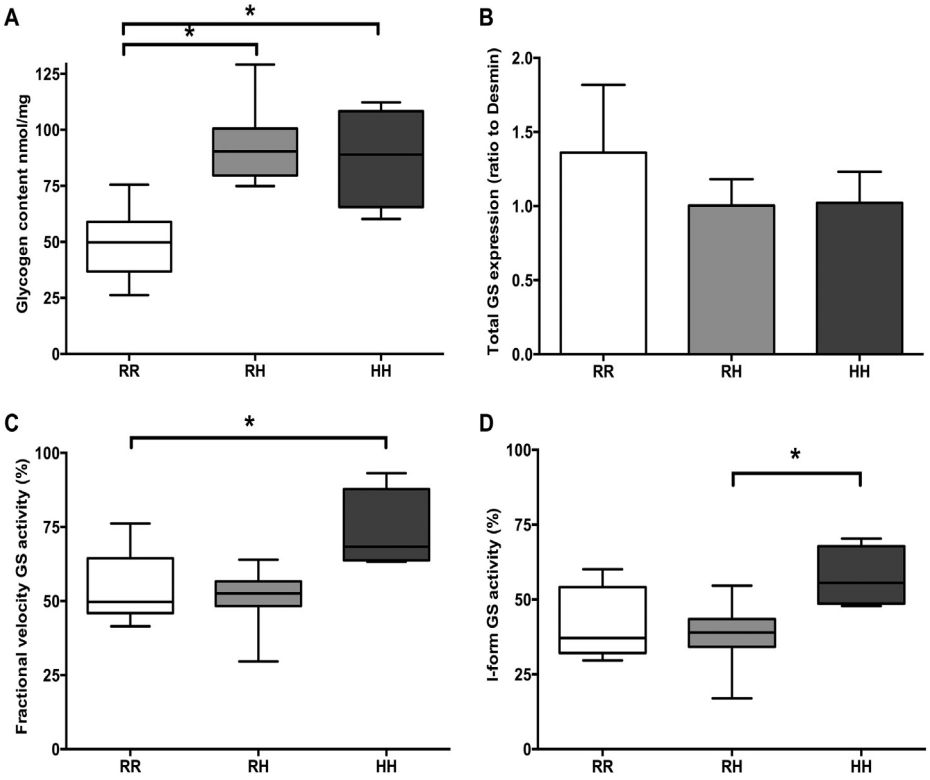

Fig. 1. A) Glycogen content of wet weight muscle samples from WT (RR)($n = 7$), PSSM1-heterozygotes (RH)($n = 8$) and PSSM1-homozygotes (HH)($n = 4$) horses (median ± min/max values, box represents interquartile range; RR vs. RH $p = 0.005$, RR vs. HH $p = 0.041$, $^*p < 0.05$). B) Total glycogen synthase expression in muscle homogenates from each of the genotypes was not significantly different ($p = 0.98$). Glycogen synthase activity shown as C) fractional velocity (FV) and D) I-form activity. There was an increase in %FV between PSSM1-homozygotes (HH) and WT controls (RR) ($p = 0.03$) and an increase in %I-form activity between heterozygotes (RH) and PSSM1-homozygotes (HH) samples ($p = 0.04$) (median ± min/max values, box represents interquartile range, RR ($n = 12$), RH ($n = 13$), HH ($n = 4$) $^*p < 0.05$).

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