Two new disorders of glycogen metabolism

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Cover illustration: PAS stain of skeletal muscle showing polyglucosan bodies in RBCK1 deficiency.

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ABSTRACT

Glycogen is a polymer of glucose and serves as a source of rapidly available energy. Glycogen synthesis is initiated by autoglucosylation of glycogenin. Two glycogenin genes, *GYG1* and *GYG2*, encode the two isoforms glycogenin-1 and -2, respectively. Glycogenin-1 is ubiquitously expressed whereas glycogenin-2 has been described as being expressed mainly in the liver but also in the heart.

This thesis describes two new disorders affecting glycogen turnover. The first disease presented as sudden cardiac arrest due to arrhythmia after exercise in a 27-year-old man. Clinical history revealed minor muscle weakness of the upper extremities. He was found to have a cardiomyopathy with abnormal glycogen storage in the heart and depletion of glycogen in the skeletal muscle. An amino acid substitution was detected from a threonine to a methionine at position 83 of glycogenin-1 that resulted in inactivated autoglucosylation. Detailed studies applying cell-free protein expression, *in vitro* glucosylation, and mass spectrometry (MS) demonstrated abolished Tyr-O-glucosylation, the initial step of the autoglucosylation. Catalytically active glycogenin-1, but not glycogenin-2, could compensate for this defect by inter-molecular glucosylation. Transcriptome and western blot analyses indicated that glycogenin-2 is mainly expressed in liver and adipose tissue and only to a minor degree in cardiac and skeletal muscle. By applying a automated analysis of glycopeptides from MS data of co-expressed glycogenin-1 and -2, we show that glycogenin-1 enhances the glucosylation of glycogenin-2.

The second disease presented as myopathy and cardiomyopathy in 10 patients from eight families. Initial symptoms were leg weakness starting in childhood or adolescence, later followed by generalized muscle weakness and cardiomyopathy. The cardiomyopathy was rapidly progressive, necessitating heart transplantation in several cases. The characteristic morphological features were inclusions of abnormal glycogen, polyglucosan,

in approximately 50% of the skeletal muscle fibers that were depleted of normal glycogen. Different truncating and missense mutations were detected in the gene *RBCK1*, coding for an E3 ubiquitin ligase. RBCK1 deficiency appears to be a common type of glycogen storage disease and is to be considered in cases with dilated cardiomyopathy.

In conclusion, we have described two new glycogenoses affecting heart and skeletal muscle and investigated the pathogenesis. The discovery of the genetic background of these novel disease entities is important for correct diagnosis, evaluation of prognosis, genetic counseling and treatment.

Keywords: Glycogen, Glycogenin, Glycogenosis, Glycogen Storage Disease, Glycosylation, Mass spectrometry

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SAMMANFATTNING PÅ SVENSKA

Energi lagras i cellen i form av polymerer av glukos s.k. glykogen. Vi beskriver två nya ärftliga ämnesomsättningssjukdomar som påverkar glykogenomsättningen. Glykogen bildas genom att proteinet glycogenin autoglukosyleras d.v.s. formar en kedja av glukos, en process som initierar glykogensyntesen.

Den första sjukdomen har fått namnet Glycogen storage disease XV (GSD 15). Fallstudien beskriver en 27-årig man som efter ett träningspass fick ett hjärtstillestånd men återupplivades. För att utreda orsaken till hjärtstilleståndet togs en hjärtbiopsi som visade upplagring av onormalt glykogen och brist på normalt glykogen medan skelettmuskelbiopsin visade endast glykogenbrist. Vi upptäckte mutationer i genen *GYG1* som kodar för proteinet glycogenin-1. De genetiska mutationerna resulterade i ett protein som inte var funktionellt. Vi studerade inbindningen av glukos vidare genom att syntetisera glycogenin-1 och levervarianten glycogenin-2 i ett cell-fritt system. Med hjälp av masspektrometriska metoder visade vi att ett funktionellt glycogenin-1, men inte glycogenin-2, kunde addera glukos på patientens defekta glycogenin-1.
Den andra sjukdomen startade som en progressiv muskelsvaghet i barndomen eller under tonåren och därtill utvecklade flera av patienterna en allvarlig hjärtproblematik med transplantation som följd. Muskelbiopsier från patienterna visade att ungefär hälften av muskelns celler hade onormala upplagringar av socker som inte liknade normalt glykogen. Med en ny teknik för att söka efter genetiska förändringar i patientens alla gener s.k. exome sekvensering kunde vi fastställa att genetiska förändringar i genen *RBCK1* orsakade sjukdomen. Totalt identifierade vi 10 patienter från åtta familjer med förändringar i *RBCK1*.

Identifikationen av den genetiska förändringen vid ärftliga sjukdomar är mycket viktigt för att kunna ställa en korrekt diagnos, förutspå sjukdomsförloppet, ge genetisk vägledning och ge en optimal behandling.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Moslemi AR, Lindberg C, Nilsson J, Tajsharghi H, Andersson B, Oldfors A, Glycogenin-1 deficiency and inactivated priming of glycogen synthesis**.** *N Engl J Med. 2010 Apr 1;362(13):1203-10.*

II. Nilsson J, Halim A, Moslemi AR, Pedersen A, Nilsson J, Larson G, Oldfors A, Molecular pathogenesis of a new glycogenosis caused by a glycogenin-1 mutation. *Biochim Biophys Acta. 2012 Apr;1822(4):493-9.*

III. Nilsson J, Halim A, Larsson E, Moslemi AR, Oldfors A, Larson G, Nilsson J, LC-MS/MS characterization of combined glycogenin-1 and glycogenin-2 enzymatic activities reveals their self-glucosylation preferences. *Biochim Biophys Acta. 2013 Nov. [Epub ahead of print].*

IV. Nilsson J, Schoser B, Laforet P, Kalev O, Lindberg C, Romero NB, López MD, Akman HO, Wahbi K, Iglseder S, Eggers C, Engel AG, Dimauro S, Oldfors A, Polyglucosan body myopathy caused by defective ubiquitin ligase RBCK1. *Ann Neurol. 2013 Jun 24. [Epub ahead of print].*

CONTENT

ABBREVIATIONS

ALT	Alanine aminotransferases
APBD	Adult polyglucosan body disease
ATP	Adenosine triphosphate
CID	Collision-induced dissociation
DMEM	Dulbecco's Modified Eagle Medium
ETD	Electron transfer dissociation
GSD	Glycogen storage disease
HEK	Human embryonic kidney
LC	Liquid chromatography
LUBAC	Linear ubiquitin chain assembly complex
m/z	Mass-to-charge ratio
MS	Mass spectrometry
MyHC	Myosin heavy chain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
TNFα	Tumor necrosis factor alpha
UDP	Uridine triphosphate
XICs	Extracted ion chromatograms

1 INTRODUCTION

1.1 Glycogen

Energy is stored within cells in the form of large glucose polymers called glycogen, which is found in the majority of organisms from unicellular bacteria and yeast to mammals. The main glycogen depots in mammals are within the skeletal muscles and the liver. Liver cells individually contain more glycogen than muscle cells, but because muscle tissue is more abundant, the muscles contain 3–4 times more total glycogen than the liver. Liver glycogen is released into the bloodstream to maintain blood glucose levels in between meals while muscle glycogen is used within each cell during muscle contraction. Glucose from muscle glycogen is readily mobilized during sudden, strenuous activity, and unlike other energy sources, it can provide energy in the absence of oxygen (Berg et al, 2002).

A glycogen granule is a large molecule, and even a single glycogen granule is visible as a dark dot on electron microscopy images. It consists of glucose linked by α1,4-glycosidic linkages and branch points that are introduced by α1,6-glycosidic linkages. The branched molecule makes glycogen soluble and increases the number of non-reducing ends accessible for degradation and resynthesis (Roach et al, 2012).

A model for the glycogen molecule granule categorizes the glucose chains within the glycogen as inner B-chains and outer A-chains (Fig. 1) (Melendez-Hevia et al, 1993). The branch points are not located on defined glucose residues, but the model suggests two branch points on each B-chain. In this model, the outer layer of A-chains would contain 50% of the total glucose residues, accessible for degradation when energy is needed. The theoretical maximum would be approximately 55 000 glucose residues. Electron microscopy of skeletal muscle shows an average glycogen diameter of 25 nm, corresponding to approximately 1600 glucose residues per glycogen granule (Graham et al, 2010).

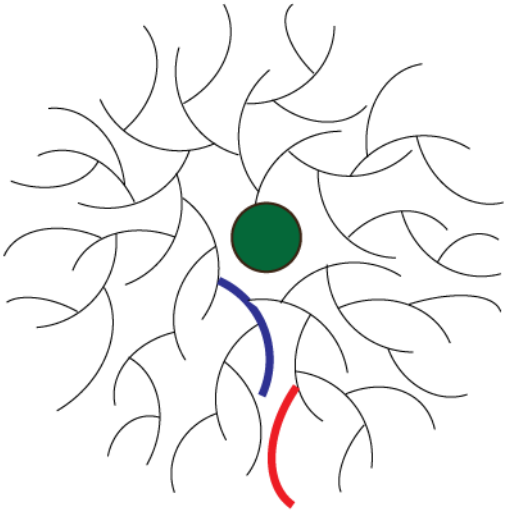

Figure 1. A glycogen molecule with glucose residues attached to a single tyrosine on the protein glycogenin (green). A model for the glycogen molecule divides the chains of glucose into inner B-chains with two branch points (blue) and outer A-chains accessible for degradation and resynthesis (red).

Glycogen contains predominately glucose but also traces of glucosamine and phosphate, and a large number of proteins associated with glycogen such as enzymes involved in the synthesis and degradation are also present (Roach et al, 2012).

1.2 Glycogen synthesis and degradation

Glycogen synthesis is initiated by the autoglucosylation of the protein glycogenin (Krisman & Barengo, 1975; Lomako et al, 1988; Pitcher et al, 1987; Pitcher et al, 1988). The self-glucosylation results in a glycogen priming chain of glucose attached to a single tyrosine that functions as a substrate for the subsequent enzymes in the glycogen synthesis (Rodriguez & Whelan, 1985; Smythe et al, 1988). The human genome contains two genes coding for glycogenin, *GYG1* and *GYG2*, which encode glycogenin-1 and -2,

respectively (Lomako et al, 1996; Zhai et al, 2000). *GYG1* is localized on chromosome 3q24 (Lomako et al, 1996), and *GYG2* is located on the X chromosome at Xp22.3 (Zhai et al, 2000). Rodents and rabbits have a single glycogenin gene, and *GYG2* is detected only in humans and higher primates (Smythe et al, 1989; Zhai et al, 2001). A gene duplication that resulted in two human glycogenin genes appears to have occurred relatively recently (LeRoith et al, 2004). The rabbit glycogenin and human glycogenin-1 sequences show a high overall homology (Fig. 2) (Lomako et al, 1996).

Figure 2. The amino acid sequence of the human glycogenin-1 protein compared to the rabbit glycogenin protein. Differences indicated in red.

Northern blot analyses have shown that glycogenin-1 is expressed in most tissues (Rodriguez & Fliesler, 1988; Viskupic et al, 1992) while glycogenin-2 is expressed in liver, heart, and pancreas in human (Mu et al, 1997).
Glycogenin-1 is a 38-kDa large protein (Pitcher et al, 1987), and glycogenin-2 is 66 kDa (Mu et al, 1997). They have an overall homology of 40–45%, but the homology over the 200 amino acids containing the catalytic region is 72% (Mu et al, 1997). Both glycogenin-1 and -2 are highly glucosylated *in vivo* and cannot be detected on western blot unless the glucose residues are cleaved off with alpha-amylase (Smythe et al, 1989). The self-glucosylation of glycogenin requires divalent cations such as Mn^{2+} or Mg^{2+} (Pitcher et al, 1988). Glycogenin uses activated UDP-glucose as a substrate and forms a glucose-O-tyrosine linkage with the hydroxyl group of a single tyrosine (Tyr-195 for glycogenin-1, (Alonso et al, 1994a) Tyr-228 for glycogenin-2, (Mu & Roach, 1998) and elongates the glucose chain by the formation of α1,4-glycosidic linkages (Fig. 3). Site-directed mutagenesis that changes the Tyr-

195 on glycogenin-1 or the Tyr-228 on glycogenin-2 into a phenylalanine results in proteins that cannot self-glucosylate but remain catalytically active (Alonso et al, 1994a; Cao et al, 1993; Lin et al, 1999; Mu & Roach, 1998; Skurat et al, 1993). Glycogenin-1 produces an oligosaccharide that contains 8–13 glucose residues (Alonso et al, 1994a; Alonso et al, 1994b; Cao et al, 1993; Lomako et al, 1990a; Lomako et al, 1990b; Romero et al, 2008). Recombinantly expressed glycogenin is glucosylated in all cells where UDP-glucose is present (Cao et al, 1993; Skurat et al, 1993; Viskupic et al, 1992). UDP-glucose is formed by the enzyme UDP-glucose pyrophosphorylase from glucose 1-phosphate and uridine triphosphate (UTP). Glycogenin-1 expressed in an *E. coli* mutant lacking this enzyme has no glucose attached (Alonso et al, 1994b).

Glycogenin-1 and -2 are biochemically similar *in vitro*, but glycogenin-1 has a higher activity and a lower Km for UDP-glucose than glycogenin-2 (Mu et al, 1997; Pitcher et al, 1988). Co-immunoprecipitation has shown that glycogenin-1 and -2 may interact with each other (Mu & Roach, 1998).

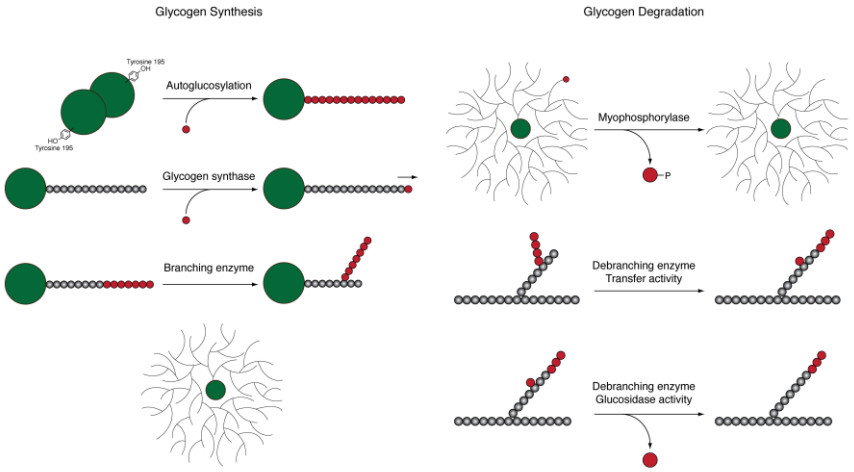

Figure 3. Schematic drawing of glycogen synthesis and degradation. Glycogenin-1 (green) self-glucosylates, glycogen synthase elongates the chain of glucose, and branching enzyme introduces branch points. Glycogen is degraded by myophosphorylase, which cleaves glucose, and by the two activities of debranching enzyme.

Glycogen synthase catalyzes the additional α1,4-glycosidic linkages in glycogen and elongates the chain of glucose molecules (Fig. 3). Glycogen synthase is encoded by two genes, *GYS1* and *GYS2*. *GYS1* is ubiquitously expressed, including in skeletal muscle and heart, while *GYS2* is restricted to liver. The regulation of glycogen synthase is complex and involves phosphorylation of the enzyme and allosteric regulation by glucose-6-phosphate, ATP, and others (Palm et al, 2013). Glycogen synthase from rabbit contains nine or more phosphorylation sites that can be phosphorylated by at least 10 different protein kinases. A branching enzyme encoded by *GBE1* transfers a chain of seven glucose residues from the end of the glucose chain and forms an α1,6-branching point (Fig. 3). When the energy demand increases within the muscle cell, glucose is released from the outer chains of the glycogen molecule by the muscle-specific enzyme myophosphorylase, encoded by the gene *PYGM*. Glucose is cleaved and phosphorylated to form glucose-1-phosphate, which enters into the glycolysis pathway. Because skeletal muscle lacks glucose-6-phosphatase, the phosphorylated glucose released from the glycogen molecule is trapped within the cell and enters the glycolysis reaction where glucose is oxidized to pyruvate, followed by ATP generation. When four glucose residues remain before a branch point, debranching enzyme, encoded by the gene *AGL*, transfers three of the glucose residues to a nearby chain, linking them with an α1,4-glycosidic linkage and subsequently cleaving the α1,6-glycosidic linkage that constitutes the branch point (Fig. 3). Debranching enzyme thus catalyzes two different enzymatic reactions in the breakdown of glycogen, the amylo-1,6-glucosidase and the oligo-1,4-glucotransferase reactions (Fig. 3). The glucose residues transferred by debranching enzyme will be accessible for myophosphorylase while the single released glucose is phosphorylated by hexokinases and enters into glycolysis.

1.3 Energy metabolism in muscle

Muscle fibers consume more energy than any other cell type. Most of the ATP is produced by the oxidation of glucose and fatty acids into carbon dioxide and water. Both glucose and fatty acids are taken up from the bloodstream (Fig. 4). When glucose is in excess, it is stored as glycogen, and under strenuous exercise, glucose is released. The metabolic processes resulting in ATP production can be divided into anaerobic glycolysis in the cytosol and the aerobic citric acid cycle and oxidative phosphorylation in the mitochondria. Glycolysis produces only two ATP from one glucose molecule but has the great advantage of being anaerobic so that oxygen is not necessary. The complete oxidation in the citric acid and oxidative

phosphorylation results in a net production of 30 ATP, but this process requires oxygen. In the absence of oxygen or during strenuous exercise, pyruvate is fermented into lactic acid. Lactic acid production takes place in skeletal muscle when energy needs outpace the ability to transport oxygen (Berg et al, 2002).

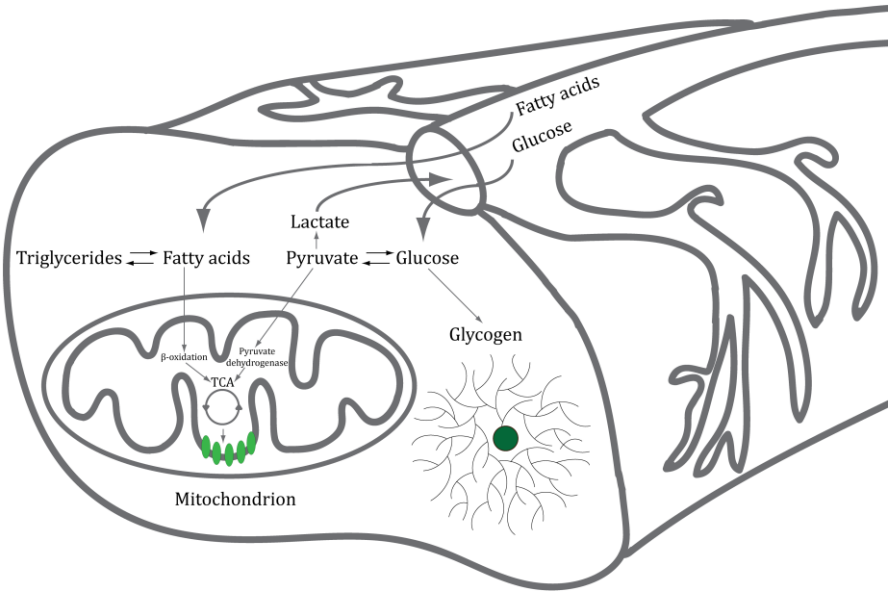

Figure 4. Energy turnover in the muscle cell. Fatty acids and glucose are taken up from the bloodstream. ATP is generated from glycolysis, the tricarboxylic acid cycle (TCA), and the electron transport chain. Excess glucose is stored as glycogen.

Human extremity skeletal muscle contains three main muscle fiber types. A muscle fiber type may be defined by the isoform of myosin heavy chain (MyHC) it expresses, its contractile speed, and its metabolism. Type I muscle fibers use oxidative metabolism and are rich in mitochondria. They are slow twitch fibers and express slow/beta cardiac myosin heavy chain (MyHC I). The fast twitch type 2 fibers are type 2A and type 2B, expressing MyHC IIa and the fastest MyHC IIx, respectively. They are rich in sarcoplasmic reticulum that allows rapid cycling of calcium ions, glycogen is abundant, and glycolytic enzymes are highly expressed. Type 2A fibers function under both aerobic and anaerobic conditions and are resistant to fatigue. Type 2B

fibers are easily fatigued and function best during bursts of anaerobic exercise (Karpati et al, 2001).

The contractile unit within the muscle fiber is the sarcomere, containing myosin (thick filament) and actin (thin filament) (Fig. 5A) (Karpati et al, 2001).

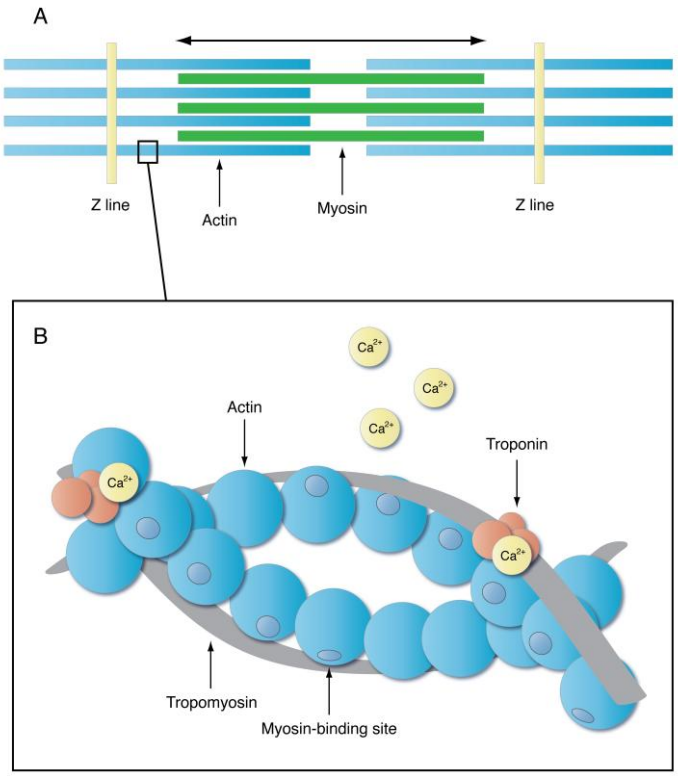

Figure 5. A: The sarcomere, the contractile unit of the muscle cell. B: Ca^{2+} ions bind to troponin and configure the tropomyosin so that the myosin-binding site on actin is revealed. Myosin binds to actin, and the sarcomere contracts. ATP is required for the myosin head to be released from actin.

At rest, the sarcolemma is at resting potential, derived from large concentrations of Na^{+} ions outside the muscle fiber, and the myosin head is

unable to interact with actin because the attachment site is blocked by tropomyosin. When a nerve impulse reaches the motor end plate, the synapse between the nerve and the muscle, acetylcholine is released, and the permeability of the sarcolemma is changed, causing influx of Na^+. An action potential is spread across the sarcolemma and deep into the fibers through t-tubules. Ca^{2+} ions, released from the adjacent sarcoplasmic reticulum, interact with the troponin complex and trigger a configuration that pulls tropomyosin away from the myosin-binding site on actin, enabling myosin to bind (Fig. 5B). Upon binding, the myosin heads bend, and as the thin filaments are dragged toward each other, the sarcomere contracts. ATP is required for the myosin head to be released from actin, for Ca^{2+} to be pumped back into the sarcoplasmic reticulum, and for the breakdown of acetylcholine in the synaptic cleft.

1.4 Glycogen storage diseases

Glycogenoses or glycogen storage diseases (GSDs) are inheritable, genetic disorders affecting glycogen turnover. The synthesis and degradation of glycogen as well as the extraction of ATP from glucose through the glycolysis pathway require enzymes, and glycogen storage diseases are caused by mutations in these enzymes (Fig. 6, Table 1). The high glucose turnover in skeletal muscle, liver, and heart makes these organs especially susceptible to defects in these enzymes. The clinical manifestations may be divided into two major groups, characterized by either exercise intolerance manifesting with pain, cramps, and myoglobinuria or fixed progressive muscle weakness (Fig. 6).

1.4.1 Glycogen storage disorders with glycogen depletion

Kollberg and co-workers described the first muscle glycogenosis with glycogen depletion in 2007 (Kollberg et al, 2007). Three affected siblings from healthy consanguineous parents had a profound glycogen deficiency in both muscle and heart. The oldest child died from sudden cardiac arrest at the age of 10.5 years, and his younger brother was investigated for muscle fatigability, hypertrophic cardiomyopathy, and an abnormal heart rate and blood pressure two years later, at the age of 11 years. Both brothers and a 2-year-old sister without symptoms had homozygous truncating mutations in *GYS1*, coding for glycogen synthase. The disease was designated as muscle glycogen storage disease type 0.

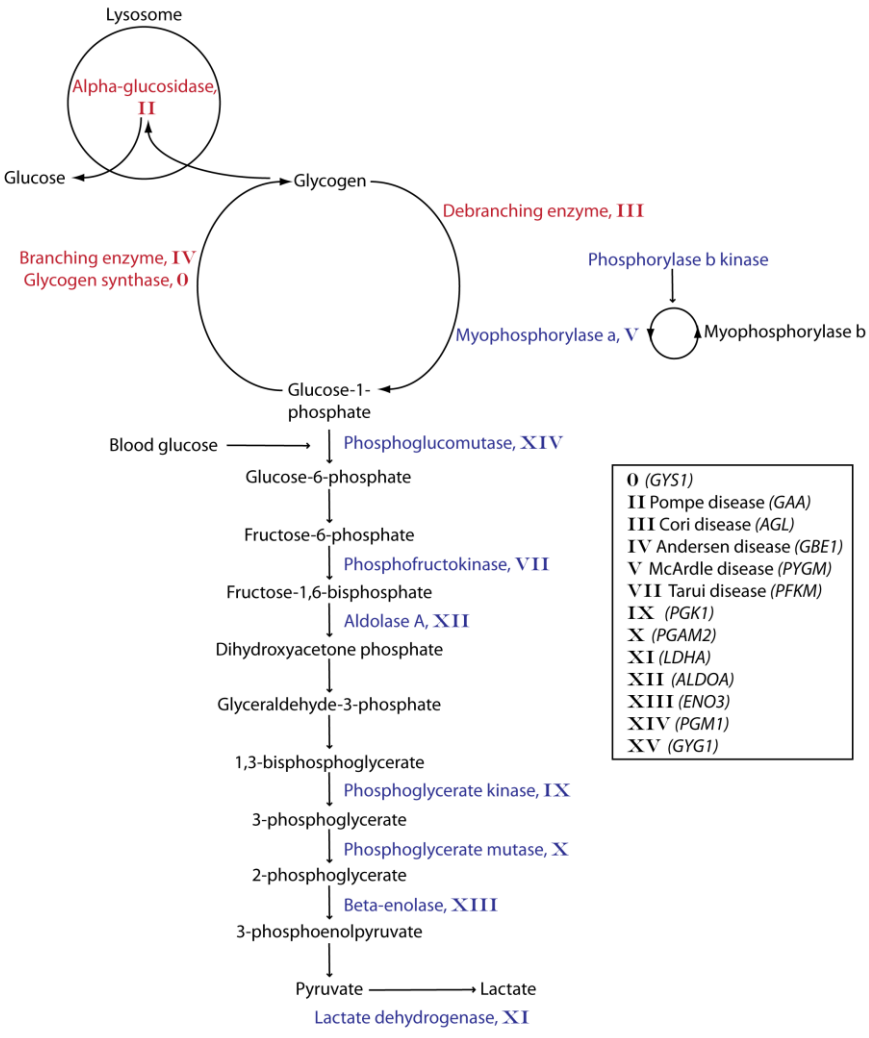

Figure 6. Schematic of glycogen turnover and glycolysis. The glycogen storage disease type (Roman numerals) and causative enzymes are shown. In general, the blue enzymes cause exercise intolerance, and the red enzymes cause progressive muscle weakness.

1.4.2 Glycogen storage disorders with glycogen accumulation

Glycogen is accumulated in several of the GSDs. These accumulations contain normally structured or abnormally structured glycogen molecules. The glycogen can be less branched, amylopectin-like, and ultra-structurally look filamentous or amorphous compared to normal glycogen. Polyglucosan bodies are glycogen accumulations that are more or less resistant to alpha-amylase digestion (Dubowitz et al, 2013).

Glycogen storage disease type II (Pompe disease) is caused by mutations in the alpha-glucosidase gene, *GAA*. Alpha-glucosidase degrades glycogen in the lysosomes by the hydrolysis of terminal non-reduced α1,4-linked glucose, and alpha-glucosidase deficiency leads to glycogen accumulation in the lysosomes. Pompe disease is divided into three clinical types: the severe infantile form with cardiomyopathy, respiratory failure, and death before 2 years of age; a juvenile form; and an adult-onset form with slowly progressive muscle weakness and, rarely, cardiomyopathy (Dubowitz et al, 2013; Karpati et al, 2001). The disease severity is related to the residual alpha-glucosidase activity (van der Ploeg & Reuser, 2008). Patients with Pompe disease may be treated with enzyme replacement therapy. Recombinant human alpha-glucosidase was previously produced in the milk of transgenic rabbits to enable some N-glycosylation (Jongen et al, 2007). The phosphorylation of the N-glycans results in mannose-6-phosphate, which targets the enzyme to the lysosome through interaction with the mannose-6-phosphate receptor, i.e., the mannose-6-phosphate pathway (Varki, 2009). Intravenous injections of the enzyme alglucosidase alfa (Myozyme®, Genzyme) produced in Chinese hamster ovarian cells are used for the treatment of Pompe patients today (Toscano & Schoser, 2013).

Glycogen storage disease type III (Forbes disease, Cori's disease) is divided into two main subgroups, type IIIa that affects liver and muscle and type IIIb that affects only liver. The causative gene is *AGL*, which encodes for the glycogen debranching enzyme, and the abnormal glycogen deposits found in this disease have very short side chains (limit dextrin). Type IIIa is associated with hypotonia, muscle weakness, hepatomegaly, short stature, dyslipidemia, and cardiomyopathy (Gazzerro et al, 2013).

Table 1. Glycogen storage disorders that affect skeletal muscle

Type	Gene	Enzyme	Clinical features
0, muscle	*GYS1*	Glycogen synthase 1 (muscle)	Muscle weakness, exercise intolerance, cardiomyopathy
II	*GAA*	Alpha-glucosidase	Severe infantile form: hypotonia, muscle weakness, cardiomyopathy, respiratory distress Juvenile and adult form: hypotonia, progressive muscle weakness
IIIa	*AGL*	Debranching enzyme	Hypotonia, muscle weakness, hepatomegaly, cardiomyopathy
IV	*GBE1*	Branching enzyme	Perinatal form: polyhydramnios, arthrogryposis, fetal hydrops, and early death Congenital/infantile form: myopathy, hypotonia muscle weakness, neuropathy; cardiomyopathy may occur Juvenile form and adult form: muscle weakness, cardiomyopathy may occur APBD: multi-organ polyglucosan body disease with nervous system involvement
V	*PYGM*	Myophosphorylase (muscle)	Exercise intolerance, cramps, fatigue, myalgia, myoglobinuria
VII	*PFKM*	Phosphofructokinase (muscle)	Exercise intolerance, cramps, myalgia, myoglobinuria, muscle weakness
IXb (VIII)	*PHKB*	Phosphorylase b kinase regulatory subunit beta	Hepatomegaly, mild muscle involvement
Ixd (VIII)	*PHKA1* (X-linked)	Phosphorylase b kinase regulatory subunit alpha (muscle)	Exercise intolerance, cramps, myalgia, myoglobinuria, muscle weakness, fatigue
IX	*PGK1* (X-linked)	Phosphoglycerate kinase	Muscle fatigue, exercise-induced cramps, myoglobinuria, neurological impairment, haemolytic anaemia
X	*PGAM2*	Phosphoglycerate mutase	Exercise intolerance, cramps, fatigue, myalgia, myoglobinuria
XI	*LDHA*	Lactate dehydrogenase	Exercise intolerance, cramps, myalgia, myoglobinuria
XII	*ALDOA*	Aldolase	Muscle weakness, muscle fatigue, haemolytic anaemia, rhabdomyolysis
XIII	*ENO3*	β-enolase (muscle)	Exercise-induced myalgia, muscle weakness, fatigue
XIV	*PGM1*	Phosphoglucomutase	Exercise intolerance, rhabdomyolysis
Other			
	PRKAG2	5'-AMP–activated protein kinase, subunit gamma-2	Cardiomyopathy, no muscle symptoms
Lafora	*EPM2A* *NHLRC1*	Laforin Malin	Progressive neurodegeneration, seizures, hallucinations, dementia

Glycogen storage disease type IV (Andersen disease) is caused by mutations in the gene *GBE1* coding for branching enzyme. Glycogen with fewer branch points and longer outer chains is accumulated in tissues with high metabolic activity such as liver and skeletal muscle. The typical presentation is liver disease, with death in early childhood (Gazzerro et al, 2013). The clinical findings differ both within and between families. The neuromuscular form is divided into four subgroups based on the amount of residual enzyme activity and clinical manifestation: the perinatal form, the congenital/infantile form, the juvenile form, and the adult form (Bruno et al, 2004). The adult forms may affect only muscle (Bruno et al, 2007) or be multisystem adult polyglucosan body disease (APBD) with both central and peripheral nervous system dysfunction (Bruno et al, 1993).

Patients with glycogen storage disease type V (McArdle disease) have myophosphorylase deficiency due to mutations in the gene *PYGM*. Excess of glycogen is visible as vacuoles at the periphery of the fibers (Dubowitz et al, 2013). The symptoms begin during childhood or adolescence with exercise intolerance that sometimes leads to rhabdomyolysis that may lead to renal failure. The breakdown of glycogen is blocked, and the cell is forced to rely on fatty acid oxidation and the uptake of glucose from the blood. These patients present a phenomenon known as the "second wind": Patients with McArdle disease experience an improved exercise tolerance after some minutes of exercise because of a switch towards oxidative metabolism of fatty acids (Vissing & Haller, 2003). A diagnostic test for McArdle disease is the ischemic forearm exercise test were lactate levels in the blood are measured. Decreased concentrations of plasma lactate in effluent blood from an arm that has exercised with blocked circulation suggest that the glycolysis is blocked (Sinkeler et al, 1986). McArdle is the most common glycolytic disorder (Haller, 2000; Nogales-Gadea et al, 2008).

Glycogen storage diseases type VII (Tarui disease; phosphofructokinase), type IX (phosphoglycerate kinase), type X (phosphoglycerate mutase), type XI (lactate dehydrogenase), type XII (aldolase A), type XIII (β-enolase), and type XIV (phosphoglucomutase) are very rare and caused by mutations in genes coding for enzymes in glycolysis. They all cause exercise intolerance and cramps, and all of them except for the cases described with aldolase A deficiency (Kreuder et al, 1996) and with lactate dehydrogenase deficiency have been associated with excess glycogen (Gazzerro et al, 2013).

Glycogen storage disease type IX is caused by mutations in several genes coding for subunits of phosphorylase b kinase, but only *PHKA1* (type Ixd) and *PHKB* (type Ixb) result in muscle involvement. *PHKA1* codes for the muscle-specific alpha subunit, and *PHKB* codes for the beta subunit of

phosphorylase b kinase, which is found in both liver and muscle (Goldstein et al, 1993). The enzyme phosphorylase b kinase phosphorylates and activates the inactive b-form of myophosphorylase into the active a-form (Cerri & Willner, 1981).

1.4.3 Other disorders with glycogen accumulation

Enzyme deficiency of the gamma-2 subunit of 5'-AMP–activated protein kinase, arising from a dominant mutation in the gene *PRKAG2*, results in accumulation of polyglucosan and hypertrophic cardiomyopathy (Arad et al, 2005).

APBD is characterized by adult-onset motor neuron involvement, sensory loss, neurogenic bladder, and dementia. Polyglucosan bodies are found in the central and peripheral nervous systems and other tissues such as skin and muscle (Robitaille et al, 1980). Many cases remain genetically undiagnosed. Mutations in the gene glycogen branching enzyme, *GBE1,* are the only known cause for APBD (Klein et al, 2004).

Lafora disease is a fatal disorder characterized by polyglucosan inclusions known as lafora bodies. It is manifested as teenage-onset myoclonus epilepsy, seizures, ataxia, and dementia. Lafora disease is caused by mutations in the gene *EPM2A*, coding for the phosphatase Laforin (Minassian et al, 1998), or the gene *NHLRC1*, coding for the E3 ubiquitin-protein ligase Malin (Chan et al, 2003). Glycogen synthase may erroneously incorporate phosphoglucose into the glycogen molecule, which is estimated to occur in 1/10,000 glucose molecules. The phosphate group is normally removed by the phosphatase Laforin, but in Lafora disease, the abnormally phosphorylated glycogen is accumulated as lafora bodies (Tagliabracci et al, 2008; Tagliabracci et al, 2011; Turnbull et al, 2010; Wang et al, 2002; Worby et al, 2006). Malin regulates the degradation of Laforin by polyubiquitination (Gentry et al, 2005), and Malin deficiency may cause lafora bodies by inhibition of the normal removal of Laforin from glycogen, with a resulting increase in levels of Laforin bound to glycogen (Tiberia et al, 2012).

Naturally occurring, dominant, gain-of-function mutations in *GYS1* that activate glycogen synthase result in polyglucosan body myopathy in horses (McCue et al, 2008). This condition may be the result of an imbalance between glycogen synthase and branching enzyme, which has been shown to cause polyglucosan bodies in transgenic mice overexpressing glycogen synthase (Raben et al, 2001).

2 AIM

The general aim of this thesis was to investigate the molecular pathogenesis of two novel glycogenoses. The specific aims were to:

- find the genetic cause and the pathogenesis of a novel disease with glycogen depletion in skeletal muscle and an accumulation of abnormal glycogen in the heart;
- use a cell-free system for co-expression of various glycogenin-1 and -2 constructs to study their self-glucosylation and inter-glucosylation;
- set up a mass spectrometry-based workflow for the characterization and quantitation of glycopeptides originating from glycogenin-1 and -2;
- investigate the *in vivo* expression of glycogenin-2; and
- use exome sequencing to find the genetic cause and to describe the clinical variability of polyglucosan body myopathy of unknown etiology.

3 PATIENTS AND METHODS

3.1 Patients and tissues

Biopsy specimens from the patient described in paper I and the two sisters described in paper IV were referred to our department because of cardiac and/or muscle disease. Samples from the remaining eight patients with different ethnic backgrounds, described in paper IV, were obtained through international collaborations. Skeletal muscle biopsies from individuals with normal muscle morphology or with glycogen depletion caused by mutations in *GYS1* (Kollberg et al, 2007), and myocardial biopsies or heart explants from patients with cardiomyopathy of other entities were used as controls. The studies were performed in accordance with the Helsinki declaration.

3.2 Skeletal muscle and myocardial biopsies

Biopsies of the deltoid, quadriceps, or cardiac muscle were mounted on cork plates with a mounting medium, fresh-frozen in liquid nitrogen, and stored at -80°C prior to enzyme histochemical and morphological studies (Dubowitz et al, 2013). Tissue specimens from skeletal or cardiac muscle were fixed in buffered glutaraldehyde and post-fixed in osmium tetroxide for electron microscopy. Ultra-thin sections of resin-embedded specimens were contrasted with uranyl acetate and lead citrate (Dubowitz et al, 2013).

3.3 Histology

3.3.1 Histochemical stains

Histochemical stains like Gomori trichrome and haematoxylin and eosin were used to visualize the tissue morphology (Dubowitz et al, 2013).

Periodic acid-Schiff (PAS) reagent was used to detect glycogen and other carbohydrates within the tissue section. Periodic acid oxidizes the carbohydrates and produces aldehyde groups. The aldehydes condense with Schiff´s reagent and yield a red color (Dubowitz et al, 2013).

3.3.2 Enzyme histochemistry

Enzyme histochemistry localizes various enzyme reactions within a tissue. Enzyme histochemical staining of myosin ATPase was used to distinguish

between fast- and slow-contracting muscle fibers. Acid preincubation (pH 4.3) inhibits myosin ATPase in the fast type 2 fibers so that only slow type 1 fibers are stained (Dubowitz et al, 2013).

Incubation for NADH-tetrazolium reductase (NADH-TR) results in a visible color in the presence of tetrazolium at the site of the enzyme activity. NADH-TR stains mitochondria and the intermyofibrillary matrix. Succinate dehydrogenase activity is specific for mitochondria (Dubowitz et al, 2013).

3.3.3 Immunohistochemistry

Immunohistochemistry is based on antibody-antigen binding. Antibodies against muscle proteins were used to localize the protein within the tissue. The antibody-protein interaction was detected with a secondary antibody with a horseradish peroxidase tag and diaminobenzidine as the chromogen (Dubowitz et al, 2013).

3.4 Tissue culturing

Muscle specimens were seeded in Petri dishes with Dulbecco's Modified Eagle Medium (DMEM), containing high glucose and glutamine levels supplemented with 10% fetal calf serum. Satellite cells spread from the biopsy, proliferated, and myoblasts were differentiated into myotubes in DMEM supplemented with 2% horse serum (Lecourt et al, 2010; Vilquin et al, 2005). The myoblasts and myotubes were histochemically stained and also harvested for DNA and RNA analyses.

3.5 Molecular genetics

3.5.1 DNA, RNA, and cDNA

Total DNA and total RNA were extracted from frozen muscle tissue or blood with commercially available kits (DNeasy Tissue Kit, DNA Blood Mini Kit, and RNeasy® Fibrous Tissue Mini Kit from Qiagen or RNAqueous®-4PCR kit from Ambion) and cDNA was produced with RNA as the template and the enzyme reverse transcriptase to yield stable copies of RNA for further analysis (Ready-To-Go™ You-Prime First-Strand Beads from Amersham Biosciences).

3.5.2 Polymerase chain reaction (PCR)

PCR was used to amplify DNA sequences with a thermostable Taq polymerase and two primers that flanked the sequence of interest. The cyclic

reaction produces thousands of millions of copies of a DNA sequence of approximately 100–1000 base pairs.

3.5.3 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis is a method in which a specific enzyme digests the mutated or wild-type allele of a fragment amplified by PCR. The digestion pattern reveals the genotype of the individual. We used the restriction enzyme Tsp45I to determine if the members of the patient's family in paper I were carriers or not for the mutations. RFLP analysis was also used to decide which alleles of glycogenin-1 were expressed at the mRNA level, i.e., detected as cDNA. Tsp451 cleaved wild-type DNA of *GYG1* into two smaller, visible bands on an agarose gel, but not the mutated c.C248T (Thr83Met) DNA that migrated as a single band. Heterozygous carriers displayed an uncleaved band from the c.C248T allele and two smaller bands from the digested wild-type allele.

3.5.4 Fragment analysis

Fragment analysis is based on the separation of PCR-amplified fragments of different lengths, detected with a fluorescently labeled primer. The Peak scanner™ software (Applied Biosystems) was used to compare the intensities of the amplified fragments, i.e., their relative abundance (Lossos et al, 2013). We used fragment analysis in paper IV to separate the amplified DNA from exon 6, with and without the 4-base-pair insertion, in patient EII:3 and showed that both alleles were expressed.

3.5.5 Sanger sequencing

Sanger sequencing is based on random incorporation of chain-terminating fluorescent A, T, G, and C dideoxynucleotides. The random introduction of terminating dideoxynucleotides results in fragments of different lengths, and after a separation on an acrylamide gel, the DNA sequence can be analyzed. Mutations were manually screened for using the Sequencing analysis software (Applied Biosystems) in which heterozygous single base pair substitutions appear as double peaks in the chromatogram.

3.5.6 Exome sequencing

Exome sequencing provides a strategy to sequence the coding regions of the entire human genome. By applying next generation sequencing, we were able to use exome sequencing to study the two sisters and their healthy mother from family A described in paper IV. We used the SureSelect XT 50Mb

Exome kit from Agilent. The entire genome was cut into small fragments of 150–200 base pairs, and the fragments were hybridized with a library of baits. The target regions were selected with magnetic beads that bound to the bait and then amplified before being loaded on an Illumina HiSeq 2000 instrument. The data identified approximately 100,000 genetic variants/individual. The lists of variants were then filtered for known polymorphisms and compared within the family to detect potentially pathogenic variants based on the assumption of a monogenic disease with an autosomal recessive Mendelian inheritance.

3.6 Protein analyses

3.6.1 Protein expression

The coding regions of a gene (copied into cDNA) were inserted into a vector with a promoter suitable for the cell in which the proteins were to be expressed. Glycogenin-1 and -2 (GenBank accession number NM_001184720 and NM_001079855, respectively) with different mutations were expressed in Chinese hamster ovarian (CHO) cells, human embryonic kidney (HEK) cells, and a cell-free *E. coli*–based expression system. The coding region of the gene was linked to the sequence for a 6xHis tag in the vector. The protein was therefore expressed with an N-terminal 6xHis tag. The tag was used to simplify protein purification and for protein detection.

3.6.2 Cell-free protein expression

A cell-free expression system is a system to express proteins *in vitro* with the use of the translational machinery extracted from cells (EasyXpress^{TM} protein synthesis, Qiagen). In comparison to protein expression in cells, the cell-free system does not require any transfection or cell culturing, and several constructs can be expressed simultaneously in a single tube. The system does not produce any posttranslational modifications of the expressed protein, and for our studies on glycogenin, it was a great advantage that the produced protein was unglucosylated.

3.6.3 Autoglucosylation and autoradiography

Glycogenin-1 and -2 were autoglucosylated *in vitro* by the addition of UDP-glucose in the presence of Mg^{2+} (Hurley et al, 2005). Radiolabeled UDP-^{14}C-glucose was used to detect glycogenin with incorporated glucose on X-ray film, i.e., via autoradiography.

3.6.4 Protein purification

Recombinantly expressed glycogenin was tagged with a 6xHis with high affinity to nickel ions. The nickel was immobilized to magnetic agarose beads, and when the protein attached to the nickel on the beads, a magnet separated the bead–protein complex from the solution (Ni-NTA magnetic agarose beads, Qiagen). The protein was washed, and for some experiments, digested with alpha-amylase while attached to the beads.

3.6.5 Western blot

Western blot was used to detect a specific protein within a solution or in a tissue lysate. All proteins within a sample were separated by polyacrylamide gel electrophoresis depending on size and transferred to a polyvinylidene difluoride (PVDF) membrane. We used the NuPAGE® SDS PAGE gel system from Invitrogen. The protein of interest was detected with an antibody against the protein or the 6xHis tag. Western blot was useful to detect specific proteins within tissues such as liver, fat, or muscle and to verify the recombinant expression in the different expression systems. It was also used to confirm autoglucosylation because the addition of glucose increased the molecular weight of glycogenin by approximately 1 kDa, enough to produce a gel shift.

The cell-free-expressed, *in vitro*-glucosylated, and His-purified glycogenin was separated on an acrylamide gel as a second purification step prior to mass spectrometry (MS) analysis.

3.6.6 Mass spectrometry and in-gel trypsinization

MS techniques produce spectra that were used to determine the protein content of a solution and to detect posttranslational modifications such as hexoses on glycogenin. Each *m/z* (mass-to-charge ratio) peak within a MS spectrum represents a unique peptide. If a peptide is selected for fragmentation, a second order spectrum results, known as a MS^2 spectrum, in which the different peaks are the peptide backbone–derived daughter ions from that peptide (Fig. 7). We used in-gel trypsinization in which the proteins were digested with trypsin in gel pieces removed from a polyacrylamide gel (Shevchenko et al, 1996). The peptides were fractioned prior to the MS analysis with a liquid chromatography column (LC). The MS instrument contains an ion source, a mass analyzer, and a detector. The ion source produces gas-phase ions from peptides, and *m/z* values of the ionized peptides are measured from their behavior in electric/magnetic fields. Modern instruments contain several mass spectrometers in series, i.e., tandem

MS or MS/MS. The precursor MS spectra were generated by a Fourier transform ion cyclotron resonance or orbitrap analyzer, and the MS^2 spectra of fragmented ions were generated by a linear ion trap quadrupole. The ionized molecules (precursor ions) were fragmented with either collision-induced dissociation (CID) or electron transfer dissociation (ETD). CID fragmentation results in the loss of monosaccharides from the peptide backbone (Huddleston et al, 1993), and ETD fragments the peptide backbone without disrupting the glycosidic bonds (Zhu et al, 2013). The Mascot algorithm was used to determine the protein identity of the fragmented peptides. The intensity of the precursor peaks from extracted ion chromatograms (XICs) were used to make a relative quantification of the different glycopeptides from glycogenin-1 and -2.

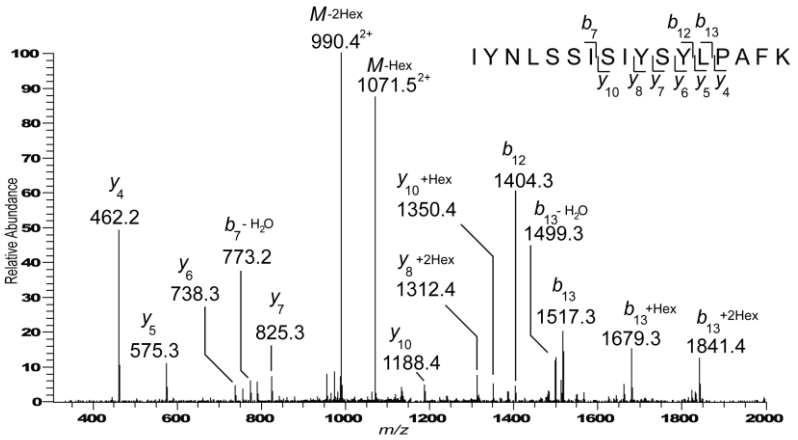

Figure 7. MS^2 spectrum showing the fragmentation pattern of the glycopeptide IYNLSSISIYSYLPAFK from glycogenin-1 attached with two hexoses. The ions produced after CID fragmentation are called b and y-ions and numbered from the N-terminal or C-terminal, respectively, of the peptide. Reproduced with permission from Biochim Biophys Acta 2012;1822:493-9.

4 RESULTS AND DISCUSSION

Glycogen storage diseases are rare disorders. In this thesis, we describe two new disorders arising from mutations in *GYG1*, which codes for the glycogen synthesis-initiating protein glycogenin-1, and *RBCK1*, which codes for an E3 ubiquitin ligase. Glycogenin-1 deficiency has been classified in OMIM (Online Mendelian Inheritance in Man, National Center of Biotechnology Information, NCBI) as glycogen storage disease XV (GSD15).

4.1 Paper I

Paper I describes a patient with glycogen depletion in the skeletal muscles and heart and accumulation of abnormal storage material in the heart (Fig. 1 and 2, respectively, paper I). The 27-year-old patient experienced dizziness and chest palpitations after exercise. He developed ventricular fibrillation, which was converted to sinus rhythm by cardiac defibrillation. Prior to the episode, the patient had normal early motor milestones even though he ran more slowly than his peers and was slightly weak in his upper arms. He was dismissed from military service due to an abnormal electrocardiogram.

4.1.1 Compound heterozygosity for mutations in *GYG1*: a novel gene associated with disease

Two mutations were detected in the gene *GYG1*, coding for glycogenin-1. A threonine-to-methionine substitution in position 83 (p.Thr83Met) was detected on one allele and a single G deletion (c.487delG) that resulted in a frameshift and a premature stop was detected on the other allele. The c.487delG nonsense mutation was found in the healthy mother and the p.Thr83Met substitution in the healthy father and in two of the patient's healthy brothers. Muscle biopsies performed on the parents, revealed no apparent glycogen depletion in accordance to an autosomal recessive inheritance. RFLP analysis indicated that Thr83Met was the only expressed allele and that the allele with the nonsense mutation probably was degraded by nonsense-mediated mRNA decay. Cultured myoblasts from the patient displayed glycogen depletion and monoallelic expression of the Thr83Met allele.

4.1.2 Lack of glucosylation of Thr83Met glycogenin-1

Glycogenin-1 from control muscle tissue was not visible on western blot because of the heavy glucosylation unless the glucose residues were cleaved off with alpha-amylase. However, glycogenin-1 from the patient was detected in muscle tissues on western blot without alpha-amylase treatment, indicating that the detected protein was unglucosylated Thr83Met glycogenin-1 (Fig 4B, paper I). Glycogenin-1 was also detected without alpha-amylase treatment in the muscle from the father, who was heterozygous for the Thr83Met mutation. We had the opportunity to add muscle specimens from a patient with glycogen synthase deficiency, to the analysis. Glycogenin-1 from the glycogen synthase-deficient patient had a higher molecular weight due to autoglucosylation than the glycogenin-1 detected in the patient and his father, indicating that Thr83Met glycogenin-1 was unable to autoglucosylate.
Recombinant glycogenin-1 was expressed in CHO cells. Wild-type glycogenin-1 autoglucosylated in this system as shown by a gel shift whereas Thr83Met did not autoglucosylate. We concluded that the Thr83Met mutation led to inactivation of the autoglucosylation of glycogenin-1. As a compensation for the lack of glycogen, the patient had a marked mitochondrial proliferation indicative of increased oxidative metabolism and a predominance of slow twitch, oxidative type 1 muscle fibers in his skeletal muscle.

4.1.3 Glycogen depletion and accumulation of PAS-positive material in the myocardium

Large vacuoles of accumulated material were detected in the myocardial biopsy. The vacuoles contained PAS-positive material that could be digested with alpha-amylase. The cytoplasm around the vacuoles was depleted of glycogen. Electron micrographs of the vacuoles showed unstructured material mixed with small vesicles, tubular structures, scattered mitochondria, and lipid droplets but no normal glycogen. Western blot analyses showed that the heart contained unglucosylated glycogenin-1 (Fig 4C, paper I). The nature of these PAS-positive vacuoles remains unclear, and a hypothesis that they originated from glycogenin-2 (*GYG2*), was investigated in paper III. No further cases with glycogen depletion due to *GYG1* mutations have been published. Either it is a very rare disorder, or affected individuals are overlooked. Sudden cardiac arrests are not always morphologically examined, and it might be possible to live an average lifespan without glycogenin-1.

In conclusion, paper I describes the first case with glycogen depletion arising from mutations in *GYG1* and impaired glycogen synthesis due to apparent defects in the autoglucosylation of glycogenin-1.

4.2 Paper II

Glycogenin-1 was mainly studied in the 1980s and 1990s, and because rabbit liver was the primary source of glycogen, all studies, even with recombinant proteins, were performed on rabbit glycogenin-1. Our studies have been performed on human glycogenin-1 (Fig. 2).

4.2.1 The cell-free expression system and gel-shift mobility assay for recombinant glycogenin-1

We have investigated the importance of threonine 83 for the catalytic activity of glycogenin-1 through recombinant expression in a cell-free *E. coli*-based expression system. This system does not contain UDP-glucose, and the expressed glycogenin-1 therefore remains unglucosylated. The recombinant glycogenin-1 was allowed to autoglucosylate *in vitro* by adding UDP-glucose and Mg^{2+}. We analyzed the autoglucosylation by western blot, autoradiography, and LC/MS/MS. *In vitro*-glucosylated wild-type glycogenin-1 expressed in the cell-free system resulted in a mobility shift on western blot of approximately 1 kDa compared to unglucosylated glycogenin-1 (Fig 2A, paper II). The molecular weight of glycogenin-1 was reduced after alpha-amylase treatment, indicating that the difference in size indeed was α-1,4-linked glucose. Approximately 50% of the glycogenin-1 molecules demonstrated this shift, suggesting that the autoglucosylation was incomplete in this system. The recombinant glycogenin-1 was analyzed with size-exclusion chromatography. Glycogenin-1 was detected as dimers and tetramers but also as larger aggregates, which might be an explanation for the partial glucosylation.

4.2.2 Incorporation of UDP-^{14}C-glucose, autoradiography and MS

In vitro glucosylation of wild-type glycogenin-1 by radiolabelled UDP-^{14}C-glucose showed incorporation of detectable levels of glucose with autoradiography (Fig. 2B, paper II). By tandem MS analyses of glycogenin-1, we detected peptides containing tyrosine 195 from glycogenin-1 modified with 2–8 hexoses (Table 1, paper II). The samples were digested with alpha-amylase prior to the analyses to reduce the *m/z* values and simplify the mass

spectrometric analyses of the glycopeptides. The results showed that the glucose moieties were selectively attached to tyrosine 195 of glycogenin-1 and never to tyrosine 187 or 197.

4.2.3 Defective autoglucosylation of Thr83Met glycogenin-1

Recombinant Thr83Met glycogenin-1 that was allowed to autoglucosylate did not show a mobility shift on western blot (Fig. 2, paper II). In addition, it did not incorporate detectable levels of UDP-^{14}C-glucose on autoradiography. We could not detect by MS a single peptide from glycogenin-1 that contained tyrosine 195 with glucose modifications, confirming that Thr83Met glycogenin-1 cannot form the glucose-O-tyrosine linkage at tyrosine 195 (Table 1, paper II).

4.2.4 The dimerization of glycogenin

Gel filtration and X-ray crystallography have shown that glycogenin-1 exists as dimers (Cao et al, 1995; Hurley et al, 2005; Smythe et al, 1990). In addition, the Tyr195Phe glycogenin-1 can glucosylate the inactive Lys85Gln glycogenin-1 (Lin et al, 1999), which suggests that one subunit transfers glucose to another. However, monomeric glycogenin-1 can autoglucosylate (Bazan et al, 2008), and structural studies have shown that the location of Tyr195 relative to the sugar-donor UDP-glucose in the active sites of the dimer prevents the autoglucosylation, at least for the attachments of the initial glucose residues, suggesting that the initial glycosylation occurs via inter-molecular catalysis (Gibbons et al, 2002) (Fig. 8). Issoglio and co-workers concluded that dimer intra-subunit glucosylation cannot significantly initiate but can complete autoglucosylation after tyrosine 195 has acquired several glucose units by inter-subunit glucosylation; i.e., both intra- and inter-subunit reaction mechanisms are necessary for maximum glucosylation (Issoglio et al, 2012).

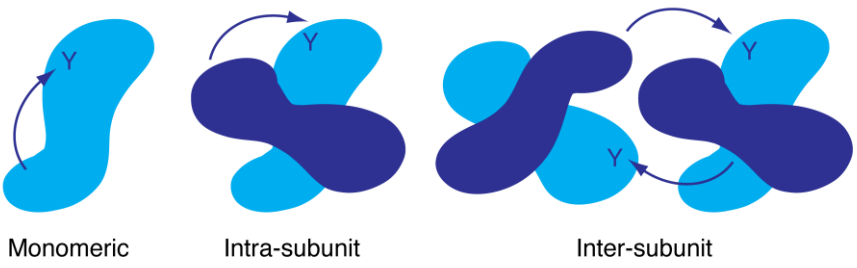

Figure 8. Potential glucosylation of glycogenin-1 at tyrosine 195 (Y).

4.2.5 Glucosylation of Thr83Met glycogenin-1 by Tyr195Phe glycogenin-1

The cell-free system allows protein expression from more than one plasmid in a single reaction. We used this method to co-express Thr83Met glycogenin-1 and a catalytically active Tyr195Phe glycogenin-1 that cannot autoglucosylate. Autoradiography showed that Tyr195Phe glucosylated Thr83Met glycogenin-1 (Fig. 4, paper II), and LC/MS/MS confirmed that the peptide containing Tyr195 was modified with at least two hexoses (Table 2, paper II). This result indicates that glucosylation can be achieved by transglucosylation aided by an enzymatically active Tyr195Phe glycogenin-1.

4.2.6 The importance of the hydroxyl group in threonine 83 for function

To test the importance of threonine 83, we exchanged it for amino acids with different side-chains (Fig. 3, paper II) and analyzed their ability to autoglucosylate in the gel-shift assay. The hydroxyl group on threonine proved to be important because Thr83Ser glycogenin-1 autoglucosylated similarly to wild type. Valine and alanine are essentially isosteric with threonine, but they do not have a hydroxyl group, and Thr83Val and Thr83Ala glycogenin-1 did not autoglucosylate. When Thr83 was changed into the bulkier amino acids methionine (in the patient), phenylalanine, or tyrosine, the switch resulted in a protein that could not autoglucosylate even though tyrosine contains a hydroxyl group.

The side chain, a hydroxyl group, and the size of the amino acid in position 83 of glycogenin-1 appear crucial for the autocatalytic activity of glycogenin-1. Thus, Thr83Met glycogenin-1 cannot autoglucosylate or catalyze the

glucose-O-tyrosine 195 linkage. However, Thr83Met glycogenin-1 is glucosylated by a catalytically active glycogenin-1.

4.3 Paper III

Glycogenin-2 was detected in human Expressed sequence tag (EST) clones from brain and breast (Mu et al, 1997). A northern blot showed that glycogenin-2 was predominately expressed in liver and was thus designated as the liver isoform (Mu et al, 1997). Glycogenin-2 was detected in a rodent cell line that later was shown to have a human karyotype, which gave rise to the question of whether rodents have glycogenin-2 (Mu et al, 1997). In this paper, we used the cell-free expression system and *in vitro* glucosylation to study the interactions between glycogenin-1 and glycogenin-2. Our studies have been performed on the most frequently expressed and catalytically active glycogenin-2 beta isoform (Mu & Roach, 1998).

4.3.1 Detection of the highest expression of glycogenin-2 in liver and fat

We plotted transcriptome sequencing data from EBI's ArrayExpress repository, and the result showed that the highest expression of glycogenin-2 was in liver and adipose tissue (Fig. S1, supplementary appendix, paper III). Western blot analyses were performed on glycogenin-1 and -2 from liver, skeletal muscle, heart, and adipose tissue (Fig. 1C, paper III). Both glycogenin-1 and -2 were detected in liver but only after alpha-amylase digestion, indicating that they are heavily glucosylated. The strongest expression of glycogenin-2 was detected in adipose tissue. Glycogenin-2 was identified in fat both before and after alpha-amylase treatment, suggesting that glycogenin-2 in fat was less glucosylated. Glycogenin-1 was expressed in liver, skeletal muscle, heart, and adipose tissue but detectable only after alpha-amylase treatment.

4.3.2 Glucosylation with 0-1 glucose residues of the majority of glycogenin-2 molecules

Autoglucosylation in the cell-free expression system resulted in glycogenin-2 that migrated like the unglucosylated Tyr228Phe glycogenin-2 on western blot. To exclude that the 6xHis tag disturbed the catalytic activity, we expressed glycogenin-2 without the tag in HEK cells, and no shift in size was detected after autoglucosylation. MS^2 spectra from LC/MS/MS analyses of the cell-free-expressed glycogenin-2 after subsequent *in vitro* glucosylation were analyzed with the protein identification software Mascot.

Unglucosylated peptides from glycogenin-2 containing tyrosine 228 were detected. The Mascot search was altered to allow modifications of glucose units (+162.0528) to perform an automated identification of glycogenin glycopeptides. Peptides from glycogenin-2 modified with 0–4 glucose residues were detected using this strategy (Table 1, paper III). Fragmentation with ETD showed that the glucose modifications indeed were on tyrosine 228. Even for these studies, the samples were treated with alpha-amylase prior to the mass spectrometric analyses to trim down the glucose chains. XICs were used to calculate the relative abundance of the various glycoforms of glycogenin-1 and -2. For glycogenin-2, unglucosylated peptides and peptides containing a single glucose residue constituted 90% of the detected peptides compared to glycogenin-1, in which the majority of peptides contained 6–7 glucose residues (Fig. 4, paper III). The maximum number of glucose molecules for glycogenin-2 was 4 compared to 8 for glycogenin-1. Samples excluded from the alpha-amylase treatment and/or prolonged autoglucosylation time from 1 to 4 hours resulted in essentially the same glucosylation profile of glycogenin-2 with the exception that small amounts of peptides containing 5 or 6 glucoses were detected. Based on these results, we concluded that the glucosylation of glycogenin-2 was insufficient to produce a gel shift on western blot.

4.3.3 Glucosylation of glycogenin-2 was enhanced by glycogenin-1

We conducted a series of experiments with co-expression of different recombinants of glycogenin-1 and -2 to study the potential transglucosylation. Glycogenin-2 alone or co-expressed with the catalytically inactive Thr83Met glycogenin-1 did not incorporate enough UDP-^{14}C-glucose to be visualized on autoradiography. However, when glycogenin-2 was co-expressed with the catalytically active Tyr195Phe glycogenin-1 or wild-type glycogenin-1, it became detectable with autoradiography. The co-expression was analyzed with MS and XICs, and glycogenin-2 co-expressed with the catalytically active Tyr195Phe glycogenin-1 or with wild-type glycogenin-1 showed a higher degree of glucosylation than wild-type glycogenin-2 alone or co-expression with the inactive Thr83Met glycogenin-1 (Fig. 5, paper III). We also showed that glycogenin-2, compared to Tyr195Phe glycogenin-1, could not glucosylate Thr83Met glycogenin-1. Glycogenin-1 and -2 has been shown to interact with each other and the glucosylation of glycogenin-2 was enhanced by the presence of glycogenin-1 (Mu & Roach, 1998).

4.3.4 The storage material in the heart of the patient in paper I

The origin of the PAS-positive vacuoles in the heart of the patient described in paper I remain enigmatic. In paper I, we present the theory that glycogenin-2 was involved in the deposition of the PAS-positive material based on a northern blot that showed a high expression of glycogenin-2 in the heart (Mu et al, 1997). However, the results in paper III demonstrated that the expression of glycogenin-2 was almost equivalent in heart and skeletal muscle. We also showed that glycogenin-2 was unable to glucosylate Thr83Met glycogenin-1 and that Thr83Met glycogenin-1 could not enhance glucosylation of glycogenin-2. It could not be excluded however that there is an overexpression of glycogenin-2 in the heart when glycogenin-1 is catalytically inactive. Stable overexpression of glycogenin-2 in Rat-1 cells resulted in a 5-fold increase in total glycogen levels (Mu & Roach, 1998). However, the size of the available myocardial biopsy specimen prevents any further studies. To overcome these limitations, we are in on-going experiments, trying to produce induced pluripotent stem cells (iPSCs) from cells originating from the muscle biopsy and differentiate them into cardiomyocytes.

In conclusion, paper III describes a novel workflow from the cell-free expression of glycogenin to the automated analyses of the MS files to detect glycopeptides. The results demonstrate that glycogenin-1 and -2 interact and that glycogenin-1 enhances the glucosylation of glycogenin-2 whereas glycogenin-2 cannot glucosylate glycogenin-1. In addition, the mass spectrometric data revealed a disparity between the average glucosylation of glycogenin-1 and -2.

4.4 Paper IV

This paper describes a new glycogen storage disease with myopathy and cardiomyopathy, arising from mutations in the gene encoding the ubiquitin ligase RBCK1.

Two middle-aged sisters were investigated for their progressive proximal muscle weakness with disease onset at adolescence. Both experienced severe muscle weakness, and only one of them remained ambulant. A rapidly progressive cardiomyopathy was diagnosed in one of the sisters at the time of investigation.

4.4.1 Identification by whole exome sequencing of mutations in the ubiquitin ligase RBCK1

Whole exome sequencing was performed on DNA from the two affected sisters and their healthy mother. The collected data were filtered, and none of the known genes associated with glycogen metabolism were mutated. The data were then filtered for genes associated with the ubiquitination pathway because of the apparent similarities between the inclusions and lafora bodies seen in Lafora disease, which in many cases are caused by mutations in the E3 ubiquitin ligase Malin. The presence of two potential pathogenic variants in the E3 ubiquitin ligase RBCK1 indicated *RBCK1* as a candidate gene.

4.4.2 *RBCK1* mutations were identified in 10 patients from eight families

In all, a total of 32 individuals with polyglucosan body myopathy were screened for *RBCK1* mutations, and 10 patients from eight families, were found to be homozygous or compound heterozygous for mutations in *RBCK1* (Fig. 1, paper IV). Each family had unique mutations (Fig. 1, paper IV). Five families (Fig. 1 B, C, E, F, and H, paper IV) with six patients carried truncating mutations, including premature stop codons and frame-shifting deletions or insertions. All patients with these deleterious mutations developed rapidly progressive cardiomyopathy with onset in adolescence. The patient in family D had a milder N-terminal missense mutation; the patient in family G had a single base insertion in the donor splice site of intron 7, which resulted in a new splice site in exon 7 and a frameshift; and the two patients in family A had a missense mutation on one allele and a premature stop on the other. The patients with missense mutations had milder clinical manifestations with either late-onset cardiomyopathy or no apparent cardiac involvement.

4.4.3 Clinical manifestations

All patients had childhood or juvenile onset of myopathy with proximal leg muscle weakness that led to difficulties in ambulation (Table 1, paper IV). Upper extremities and facial muscles were relatively unaffected even though four had ptosis and three had scoliosis. Four patients had growth retardation. Most of the patients that developed cardiomyopathy had an alarmingly rapid progression. Four patients were transplanted after cardiac failure, and one died due to cardiorespiratory failure at the age of 20 years. Two patients had liver involvement with storage of polyglucosan, one of whom had liver enlargement and raised levels of alanine aminotransferases (ALT), an indication for liver disease. Three additional patients had elevated levels of

ALT. Type 1 diabetes, sarcoidosis, and gluten intolerance occurred in isolated cases (Table 1, paper IV). One patient had frequent episodes of pharyngitis, lymphadenopathy in childhood, granulomatous tonsillitis, enteritis, psoriasis, and osteoporosis, but immunological investigations were normal.

4.4.4 Muscle morphology

Approximately 50% of the muscle fibers had inclusions of PAS-positive material that was incompletely digested by alpha-amylase. The surrounding cytosol was depleted of glycogen (Fig. 2, paper IV). The inclusions were positive for the ubiquitin-binding protein sequestosome-1 (p62), which indicates that they were marked for proteasomal or lysosomal degradation. Electron micrographs of the storage material showed granular and partially fibrillar structures of variable electron density, characteristic features for polyglucosan (Fig. 2D, and supplementary appendix Fig. 1, paper IV). Virtually all cardiomyocytes contained polyglucosan. Thus, paper IV describes that RBCK1 deficiency is a novel and frequent cause of polyglucosan storage myopathy associated with progressive muscle weakness and cardiomyopathy.

4.4.5 Proteins that interact with RBCK1

RBCK1 has transcriptional (Tokunaga et al, 1998) as well as ubiquitin ligase activities (Tatematsu et al, 2008) through interactions with several proteins such as the phosphatase EYA1 and the iron regulatory protein 2 (Landgraf et al, 2010; Yamanaka et al, 2003). EYA1 is enriched in fast twitch fibers, and overexpression of EYA1 in slow twitch muscles activates genes of the glycolytic metabolic pathway (Grifone et al, 2004). The paralogous *EYA4* is associated with dilated cardiomyopathy and sensorineural hearing loss (Schonberger et al, 2005). Together with the proteins Hoip-1 and Sharpin, RBCK1 forms the linear ubiquitin chain assembly complex, LUBAC (Ikeda et al, 2011; Nakamura et al, 2006). LUBAC forms a unique polyubiquitination chain in which the carboxy-terminal glycine of one ubiquitin molecule forms a peptide bond with the amino-terminal methionine of another (Kirisako et al, 2006). Ubiquitination by LUBAC is a signal for proteasomal degradation (Kirisako et al, 2006). In addition, LUBAC is a critical regulator of the NF-κB pathway and therefore associated with the immune system (Ikeda et al, 2011; Nakamura et al, 2006).

4.4.6 Animal models of RBCK1 deficiency

RBCK1 studies on animal models have produced divergent results. Morpholino-mediated knockdown of *Rbck1* in zebrafish led to a BOR syndrome-like phenotype with defects in ear and branchial arch formation (Landgraf et al, 2010). A *Rbck1* knockout mouse was produced to study the immune system; however, the mouse showed no overt phenotype, and in contrast to primary hepatocytes isolated from the *Rbck1* knockout mouse that had impaired TNFα-induced NF-κB activation, the TNFα-induced NF-κB activation in the mouse was not completely abolished (Iwai, 2011; Tokunaga & Iwai, 2012). We have looked for accumulated carbohydrates in fresh-frozen tissues from the heart of 6-month-old homozygous *Rbck1* knocked-out mice and could detect polyglucosan in the myocardium (unpublished data).

4.4.7 RBCK1 and immunodeficiency

Boisson and co-workers described two young siblings and one unrelated child with severe immunodeficiency due to mutations in *RBCK1* (Boisson et al, 2012). Two of the children died from sepsis at ages 8 and 3.5, and the third child died from sudden respiratory distress at 4 years of age. Inclusions of polyglucosan were identified in skeletal muscle, heart, and liver. None of our patients had sepsis or auto-inflammation. One patient had some signs of immunologic disturbance like psoriasis, non-specific granulomatous disease, and frequent episodes of laryngitis during childhood. However, immunological investigations did not reveal any dysfunction.

5 CONCLUSION

The conclusions from this thesis are:

- Glycogen depletion in the skeletal muscle of a patient with a new type of glycogenosis (GSD type XV) with myopathy and cardiomyopathy was discovered to be due to mutations in *GYG1*, coding for the glycogen synthesis-initiating protein glycogenin-1. The detected Thr83Met glycogenin-1 substitution resulted in a loss of autoglucosylation activity.

- Gel-shift assays and mass spectrometric analyses of recombinant, autoglucosylated Thr83Met glycogenin-1 revealed that the patient's glycogenin-1 was unable to form the initiating glucose-O-tyrosine linkage. However, Thr83Met glycogenin-1 was glucosylated by a catalytically active glycogenin-1 molecule. The side chain, a hydroxyl group, and the size of the amino acid in position 83 of glycogenin-1 proved to be crucial for the autocatalytic activity.

- LC-MS/MS analyses of cell-free-expressed, autoglucosylated glycogenin-2 or co-expression of glycogenin-1 and -2 revealed that catalytically active glycogenin-1 enhanced the glucosylation of glycogenin-2 through the formation of longer glucose chains. However, glycogenin-2 could not glucosylate Thr83Met glycogenin-1. We also showed that the highest expression of glycogenin-2 was within liver and fat. Compared to liver glycogenin-2, glycogenin-2 from adipose tissue appeared to be sparsely glucosylated.

- Mutations in the gene *RBCK1* that codes for an E3 ubiquitin ligase were discovered to be causative for a previously genetically undefined glycogenosis with cardiomyopathy and myopathy with accumulation of polyglucosan.

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