Proteomic characterisation of polyglucosan bodies in skeletal muscle in RBCK1 deficiency

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Abstract

Aims: Several neurodegenerative and neuromuscular disorders are characterised by storage of polyglucosan, consisting of proteins and amylopectin-like polysaccharides, which are less branched than in normal glycogen. Such diseases include Lafora disease, branching enzyme deficiency, glycogenin-1 deficiency, polyglucosan body myopathy type 1 (PGBM1) due to RBCK1 deficiency and others. The protein composition of polyglucosan bodies is largely unknown.

Methods: We combined quantitative mass spectrometry, immunohistochemical and western blot analyses to identify the principal protein components of polyglucosan bodies in PGBM1. Histologically stained tissue sections of skeletal muscle from four patients were used to isolate polyglucosan deposits and control regions by laser microdissection. Prior to mass spectrometry, samples were labelled with tandem mass tags that enable quantitative comparison and multiplexed analysis of dissected samples. To study the distribution and expression of the accumulated proteins, immunohistochemical and western blot analyses were performed.

Results: Accumulated proteins were mainly components of glycogen metabolism and protein quality control pathways. The majority of fibres showed depletion of glycogen and redistribution of key enzymes of glycogen metabolism to the polyglucosan bodies. The polyglucosan bodies also showed accumulation of proteins involved in the ubiquitin-proteasome and autophagocytosis systems and protein chaperones.

Conclusions: The sequestration of key enzymes of glycogen metabolism to the polyglucosan bodies may explain the glycogen depletion in the fibres and muscle function impairment. The accumulation of components of the protein quality control systems and other proteins frequently found in protein aggregate disorders indicates that protein aggregation may be an essential part of the pathobiology of polyglucosan storage.

KEYWORDS
glycogen metabolism, glycogen storage disease, polyglucosan, protein aggregation

Abbreviations: LMD, laser microdissection; LUBAC, linear ubiquitin assembly chain complex; MS, mass spectrometry; PAS, periodic acid-Schiff; PGBM1, polyglucosan body myopathy type 1; PGBM2, polyglucosan body myopathy type 2.

[Correction added on 6 October 2021, after first online publication: Peer review history statement has been added.]

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INTRODUCTION

Glycogen is a large macromolecule forming a particle of about 20 nm in diameter. It is composed of long chains of glucose units with $\alpha$-1,4-glucosidic linkages, which are branched through $\alpha$-1,6-glucosidic linkages. Glycogen has a protein core of glycogenin, which acts as a primer for de novo glycogen synthesis. Polyglucosan refers to abnormal amylopectin-like polysaccharides, which are less branched than normal glycogen and may aggregate into polyglucosan bodies. It includes various proteins and is, like normal glycogen, stained with periodic acid–Schiff (PAS) histochemistry. It is resistant to digestion with $\alpha$-amylase to a variable extent. Polyglucosan has a fibrillar structure under the electron microscope and can be seen in normal ageing as polyglucosan bodies in the heart and as corpora amylacea in the central nervous system.

Diseases involving accumulation of polyglucosan are frequently inborn errors of metabolism caused by defects in the metabolic pathways of glycogen synthesis or degradation. The different diseases manifesting with polyglucosan storage in muscle are summarised in Table 1.2–50

Loss of function mutations of the RBCK1 gene is associated with polyglucosan body myopathy 1 (PGBM1), which is a rare, frequently fatal, autosomal recessive disorder characterised by accumulation of polyglucosan in several tissues, skeletal myopathy and cardiomyopathy leading to loss of ambulation and heart failure in many cases.11 Some individuals develop a severe immune system dysregulation with immunodeficiency and autoinflammation.12–16 RBCK1 encodes RANBP-type and C3HC4-type zinc finger-containing 1 or heme-oxidised IRP2 ubiquitin ligase 1 (HOIL-1). The RBCK1 protein is an E3 ubiquitin ligase known to function as a subunit of the linear ubiquitin chain assembly complex (LUBAC) that is a regulator of NF-kB and the immune system. In the context of myopathy and cardiomyopathy, the role of RBCK1 and the mechanisms leading to polyglucosan deposits when RBCK1 function is affected is unknown.

Polyglucosan bodies are thus associated with several clinically and genetically different diseases, but the massive storage of polyglucosan is a common morphological hallmark and considered to be central to the pathobiology of the diseases.17 The protein composition is important to explore in order to understand the pathobiology of the polyglucosan body diseases. Knowledge about the composition of polyglucosan bodies with regard to polysaccharides and proteins is also essential when designing pharmacological interventions to prohibit or reduce the polyglucosan burden of the affected cells.

We have performed protein analysis of the storage material in four unrelated patients with RBCK1 deficiency by combining laser microdissection (LMD) and mass spectrometry (MS), immunohistochemical investigations and western blot analyses. We demonstrate that the protein component of the inclusions is mainly composed of proteins related to glycogen metabolism and cellular quality control mechanisms. Sequestration to the polyglucosan bodies of key enzymes involved in glycogen metabolism explains the glycogen depletion seen in the muscle fibres with polyglucosan bodies and may also explain the impaired muscle function. The increase of the protein quality control systems shows similarities with protein aggregate diseases of muscle and neurodegenerative diseases indicating that the pathogenesis of polyglucosan involves a component that may impair protein degradation, which may have implications for treatment.

MATERIAL AND METHODS

Patients

Four patients (P1–P4) diagnosed with PGBM1 and with known mutations in RBCK1 and histological hallmarks of polyglucosan disease were included in this study (Figure 1 and Table 2). The clinical features of the patients have been previously described.11,15 Muscle biopsy specimens were obtained by open biopsy and frozen in isopentane chilled in liquid nitrogen. As a positive control for autophagy, we included a patient with chloroquine myopathy. Two patients with inclusion body myositis (IBM) and numerous protein aggregates associated with rimmed vacuoles were included as disease controls for immunohistochemistry analysis of glycogen related proteins and p62.

Laser microdissection and quantitative mass spectrometry

The entire workflow for LMD-MS is summarised in Figure 2. Ten micrometre thick, frozen skeletal muscle sections were placed on polyethylene naphthalate (PEN) MembraneSlides 1.0 (Zeiss). The sections were stained with an adapted protocol for PAS. In short, samples were incubated (all reagents cooled on ice) in 70% ethanol for 10 s, 0.5% periodic acid (Sigma-Aldrich, 77310) for 20 s, dehydrated in an ethanol series (70% for 20 s, 95% for 20 s, 100% two times for 20 s) and air-dried for 30 min. Samples were stored in air-tight containers at –80°C until used for LMD. A total
area of 50,000 μm² of polyglucosan bodies (in average 150 individual tissue regions) was isolated from each patient’s muscle biopsy using a PALM Microdissection System (Zeiss) and collected in AdhesiveCap 500 opaque tubes (Zeiss). A corresponding total area and number of regions of tissue with normal morphology by PAS staining were dissected from the same sections for each patient and collected separately for comparison with polyglucosan. Samples from P1–P3 were subjected to repeated LMD-MS analysis, whereas P4 samples were analysed once due to limited amounts of sample. Laser microdissected samples were directly treated with trypsin as previously described,18 with minor modifications and peptides were labelled using tandem mass tag (TMT) isobaric mass tagging reagents (Thermo Scientific) to enable the identification of differentially abundant proteins in polyglucosan and control tissue for each patient. The proteomic profiling is described in detail in the Supporting Information.

### Morphological and immunohistochemical analyses

Cryostat sections (8 μm) of fresh-frozen muscle tissue were analysed by standard histochemical technique for PAS staining. For diastase treatment, a 30-min incubation in phosphate buffered saline (pH 6.0) with or without 0.1% diastase was applied followed by PAS staining.

| Gene   | Protein                                      | Disease and clinical features                                                                 |
|--------|----------------------------------------------|---------------------------------------------------------------------------------------------|
| RBCK1  | Heme-oxidised IRP2 ubiquitin ligase 1        | PGBM1 (OMIM #615895): Early onset progressive muscle weakness and cardiomyopathy sometimes associated with severe immune system dysregulation and autoinflammation. |
| GYG1   | Glycogenin-1                                 | GSD15 (OMIM #613507): Dilated cardiomyopathy with minor skeletal myopathy                   |
| GBE1   | Branching enzyme                             | PGBM2 (OMIM #616199): Slowly progressive muscle weakness with onset in adulthood            |
| EPM2A  | Laforin                                      | GSD4 (OMIM #232500)                                                                            |
| EPM2B  | Malin                                        | Andersen disease: Early onset rapidly progressive liver disease                              |
| PFKM   | Phosphofructokinase                          | Neuromuscular forms: perinatal (arthrogryposis, fetal hydrops and early death), congenital, juvenile or adult onset of myopathy/neuropathy, and may include cardiomyopathy |
| PRKAG2 | Gamma subunit of AMP-activated protein kinase | APBD (OMIM #263570): Adult onset of neurogenic bladder, spastic gate, peripheral neuropathy and mild cognitive impairment |
| KLHL24 | Kelch-like protein 24                        | GSD7 (OMIM #232800): Muscle weakness, exercise intolerance and compensated hemolytic anaemia. Polyglucosan only in some cases |
|        |                                              | (OMIM #600858): Familial hypertrophic cardiomyopathy with Wolf-Parkinson-White syndrome, CMH6 Polyglucosan only in cardiac muscle |

Abbreviations: APBD, adult polyglucosan body disease; CMH, familial hypertrophic cardiomyopathy; GSD, glycogen storage disease; PGBM, polyglucosan body myopathy.
For immunohistochemistry, cryostat sections (8 μm) were fixed in acetone for 10 min, air-dried and further processed in a Dako Autostainer using the Dako EnVision FLEX High pH kit (Agilent). Primary antibodies (Table S1) were applied for 1 h.

### Immunoblot analysis

Protein was extracted from cryostat sections (10 μm) in SDS-urea buffer (125 mM Tris–HCl, 4% SDS, 4-M urea, 10% glycerol, 100-mM DTT, 0.001% bromophenol blue (pH 8.0) at 95°C for 10 min and samples cleared by centrifugation (14,000 rcf, 5 min). For alfa-amylase treatment preceding glycogenin-1 analysis, samples were incubated for 1 h at 37°C in phosphate-buffered saline (PBS) pH 6.5 with 50 units/ml of alfa-amylase (A0521, Sigma-Aldrich) or without enzyme and subsequently extracted as above. Protein concentration was determined with the Pierce 660-nm protein assay, and 10 μg was loaded per well on NuPAGE 4%–12% Bis-Tris gels (Thermo Scientific) followed by transfer to PVDF membranes and subsequent Coomassie staining of the gels. Membranes were blocked with 5% skim milk and incubated with primary antibodies (Table S1) over night at 4°C. Protein bands were visualised with HRP-conjugated secondary antibodies and SuperSignal West Femto substrate (Thermo Scientific).
RESULTS

To identify the major protein components of polyglucosan bodies in PGBM1 patients, we applied LMD-MS as outlined in Figure 2. We identified 303 proteins of which 51 were accumulated by an average ratio of 1.2 or more in polyglucosan bodies of all patients and 31 proteins that were reduced in polyglucosan bodies by an average ratio of 0.6 or less (Data S1).

The 51 accumulated proteins were assigned to groups based on their main cellular function (Table 3). Analysis of protein–protein interaction networks using the STRING database showed a functional interplay of the accumulated proteins with interaction clusters forming largely in line with the protein groups (Figure S1). A substantial part of these proteins were glycogen-related including enzymes with anabolic (e.g., glycogen synthase) or catabolic (e.g., myophosphorylase) activity. Some key enzymes of glycolysis (e.g., muscle phosphofructokinase) were also enriched in the polyglucosan bodies. Many of the remaining accumulated proteins were associated with cellular quality control having key functions in the ubiquitin-proteasome and autophagy degradation pathways or acting as protein chaperones (Table 3).

Most of the proteins that were reduced in the polyglucosan bodies compared to normal appearing sarcoplasm were components of the sarcomere and cytoskeleton (Table 4).

The MS results for 17 candidate proteins that were identified as accumulated by LMD-MS were validated by immunohistochemistry. To confirm the accumulation of the proteins is associated with polyglucosan, serial sections of muscle tissue were stained with PAS and antibodies to the 17 accumulated proteins. Protein expression and antibody specificity were further examined by immunoblot analysis of patient and control muscle homogenates.

Immunohistochemical staining of several proteins involved in glycogen metabolism such as glycogenin-1 (GYG1), glycogen synthase (GYS1), myophosphorylase (PYGM), debranching enzyme (AGL) and laforin (EPM2A) colocalised to PAS-positive polyglucosan aggregates with prominent staining corresponding to the polyglucosan bodies and depletion of the same enzymes in the surrounding sarcoplasm (Figure 3). Glycolysis-related proteins such as
| Protein | UniProt | Gene | MW | UP | P1 | P2 | P3 | P4 | p   | PAR |
|--------|---------|------|----|----|----|----|----|----|-----|-----|
| Glycogen metabolism | | | | | | | | | | |
| Glycogenin-1 | P46976 | GYG1 | 39 | 4 | 5.5 | 17.8 | 5.7 | 6.9 | 0.01 | 9.3 |
| Laforin | O95278 | EPM2A | 37 | 2 | 6.1 | 12.3 | 9.2 | 4.7 | 0.00 | 8.5 |
| Glycogen phosphorylase, muscle form | P11217 | PYGM | 97 | 25 | 3.8 | 7.5 | 6.5 | 5.3 | 0.00 | 5.8 |
| Glycogen [starch] synthase, muscle | P13807 | GYS1 | 84 | 4 | 4.4 | 4.4 | 5.4 | 3.2 | 0.00 | 4.5 |
| Glycogen debranching enzyme | P35573 | AGL | 175 | 6 | 2.1 | 4.2 | 1.9 | 2.4 | 0.01 | 2.7 |
| UTP--glucose-1-phosphate uridylyltransferase | Q16851 | UGP2 | 57 | 9 | 2.2 | 1.5 | 1.3 | 1.4 | 0.03 | 1.6 |
| Glycolysis | | | | | | | | | |
| 6-Phosphofructokinase, muscle type | P08237 | PFKM | 85 | 10 | 1.7 | 1.7 | 1.7 | 2.1 | 0.00 | 1.7 |
| Glyceraldehyde-3-phosphate dehydrogenase | P04406 | GAPDH | 36 | 9 | 1.1 | 1.5 | 1.5 | 1.8 | 0.05 | 1.5 |
| Glucose-6-phosphate isomerase | P06744 | GPI | 63 | 2 | 1.5 | 1.5 | 1.5 | 1.3 | 0.00 | 1.5 |
| Chaperone activity | | | | | | | | | |
| Heat shock protein HSP 90-alpha | P07900 | HSP90AA1 | 85 | 5 | 2.2 | 1.7 | 1.6 | 1.8 | 0.00 | 1.8 |
| Heat shock protein beta-1 | P04792 | HSPB1 | 23 | 7 | 1.8 | 1.6 | 2.1 | 1.5 | 0.01 | 1.8 |
| Alpha-crystallin B chain | P02511 | CRYAB | 20 | 7 | 2.6 | 1.3 | 1.5 | 1.3 | 0.06 | 1.8 |
| Heat shock protein HSP 90-beta | P08238 | HSP90AB1 | 83 | 5 | 1.1 | 2.2 | 1.4 | 2.4 | 0.06 | 1.7 |
| Heat shock 70-kDa protein 1A/1B | P08107 | HSPA1A | 70 | 7 | 1.4 | 1.6 | 1.4 | 1.9 | 0.01 | 1.5 |
| Heat shock cognate 71-kDa protein | P11142 | HSPA8 | 71 | 7 | 1.6 | 1.3 | 1.2 | 1.2 | 0.02 | 1.3 |
| Ubiquitin-proteasome system and autophagy | | | | | | | | | |
| Sequestosome-1 (p62) | Q13501 | SQSTM1 | 48 | 7 | 4.5 | 9.4 | 8.1 | 1.4 | 0.04 | 6.5 |
| Polyubiquitin-C (ubiquitin) | P0CG48 | UBC | 77 | 3 | 5.9 | 6.0 | 5.5 | 6.1 | 0.00 | 5.9 |
| UV excision repair protein RAD23 homologue B | P54727 | RAD23B | 43 | 2 | 4.7 | 3.7 | 3.5 | 4.7 | 0.00 | 4.2 |
| Proteasome subunit alpha type-1 | P25786 | PSMA1 | 30 | 1 | 5.6 | 2.8 | 2.9 | 2.5 | 0.01 | 3.5 |
| 26S protease regulatory subunit 7 | P35998 | PSMC2 | 49 | 1 | 6.4 | 3.1 | 2.1 | 1.2 | 0.07 | 3.2 |
| 26S proteasome non-ATPase regulatory subunit 13 | Q9UNM6 | PSMD13 | 43 | 1 | 5.3 | 2.8 | 1.7 | 1.9 | 0.03 | 2.9 |
| Proteasome subunit beta type-7 | Q99436 | PSMB7 | 30 | 1 | 3.1 | 2.6 | 2.4 | 3.4 | 0.00 | 2.8 |
| Proteasome subunit alpha type-4 | P25789 | PSMA4 | 29 | 1 | 4.1 | 3.2 | 1.8 | 2.0 | 0.02 | 2.8 |
| Proteasome subunit beta type-1 | P20618 | PSMB1 | 26 | 1 | 3.6 | 1.8 | 4.1 | 1.3 | 0.05 | 2.7 |
| Transitional endoplasmic reticulum ATPase | P55072 | VCP | 89 | 5 | 2.4 | 2.9 | 2.1 | 2.3 | 0.00 | 2.4 |
| Proteasome subunit alpha type-5 | P28066 | PSMA5 | 26 | 1 | 3.2 | 2.1 | 1.7 | 2.7 | 0.01 | 2.4 |
| COP9 signalosome complex subunit 1 | Q13098 | GPS1 | 56 | 1 | 2.4 | 2.0 | 2.7 | 2.1 | 0.00 | 2.3 |
| NSFL1 cofactor p47 | Q9UNZ2 | NSFL1C | 41 | 1 | 2.3 | 1.5 | 2.6 | - | 0.04 | 2.1 |
| Proteasome subunit alpha type-3 | P25788 | PSMA3 | 28 | 1 | 2.4 | 2.1 | 1.8 | 1.5 | 0.01 | 2.0 |
| Ubiquitin carboxyl-terminal hydrolase isozyme L1 | P09936 | UCHL1 | 25 | 4 | 2.1 | 1.9 | 1.3 | 2.5 | 0.02 | 1.9 |
| Kelch-like protein 41 | O60662 | KHL41 | 68 | 3 | 1.4 | 1.1 | 1.3 | 1.2 | 0.03 | 1.3 |
| Proteasome subunit beta type-6 | P28072 | PSMB6 | 25 | 3 | 1.4 | 1.2 | 1.2 | 1.1 | 0.02 | 1.2 |
| Redox homeostasis and oxidative stress | | | | | | | | | |
| Superoxide dismutase [Mn], mitochondrial | P04179 | SOD2 | 25 | 2 | 2.2 | 1.8 | 1.3 | 1.7 | 0.01 | 1.7 |
| Peroxiredoxin-1 | Q66830 | PRDX1 | 22 | 4 | 1.6 | 1.9 | 1.6 | 1.8 | 0.00 | 1.7 |
| Thioredoxin | P10599 | TXN | 12 | 1 | 1.4 | 1.5 | 2.4 | 1.2 | 0.07 | 1.7 |
| Peroxiredoxin-2 | P32119 | PRDX2 | 22 | 4 | 1.6 | 1.1 | 1.3 | 1.2 | 0.04 | 1.3 |
| Structural proteins | | | | | | | | | |
| Desmin | P17661 | DES | 54 | 20 | 1.9 | 2.8 | 2.9 | 1.9 | 0.01 | 2.4 |
| Tubulin alpha-4A chain | P68366 | TUBA4A | 50 | 5 | 1.6 | 1.8 | 1.3 | 1.7 | 0.01 | 1.6 |
| Tubulin beta-3 chain | Q13509 | TUBB3 | 50 | 1 | 1.7 | 1.8 | 1.3 | 1.5 | 0.01 | 1.5 |
| Tubulin beta-4B chain | P68371 | TUBB4B | 50 | 2 | 1.5 | 1.1 | 1.8 | 1.1 | 0.10 | 1.4 |

(Continues)
After treatment with alpha amylase, the pattern was consistent with alpha amylase to mobilise free glycogenin-1 before gel electrophoresis of glycogenin-1, it is therefore necessary to digest the glycogen granules because it is the primer for glycogen synthesis. For western blot analysis on human muscle, the glycogenin-1 protein is bound to glycogen granules and determined by western blot analysis (Figure 3). To be more abundant in patient muscle compared to control as an overall increase of the proteins. Laforin (EPM2A), however, appeared to be more abundant in patient muscle compared to control as determined by western blot analysis (Figure 3). In addition to the localization of glycogenin-1 to polyglucosan bodies, the molecular state of glycogenin-1 appeared to be different in PGBM1 patient muscle compared with control muscle. In normal human muscle, the glycogenin-1 protein is bound to glycogen granules because it is the primer for glycogen synthesis. For western blot analysis of glycogenin-1, it is therefore necessary to digest the glycogen with alpha amylase to mobilise free glycogenin-1 before gel electrophoresis. After treatment with alpha amylase, the pattern was similar in patient and control muscles (Figure 3). Without alpha amylase treatment, the glycogenin-1 appeared as multiple bands of different sizes in patient muscle but not in control muscle where only a faint band of normal size glycogenin-1 was present. This finding indicates that some of the glycogenin-1 in patient muscles were linked to polysaccharides of various length and may be separated by size by gel electrophoresis, unlike the normal muscle where nearly all glycogenin-1 is embedded in glycogen granules that are too large to enter into the gel.

A selection of cellular quality control proteins involved in protein degradation (ubiquitin, p62, VCP, 20S proteasome subunits, KLHL41) or chaperoning functions (HSPB1, HSP70, CRYAB, RAD23B) had accumulated in the polyglucosan according to the MS results and was verified by immunohistochemistry to be enriched in polyglucosan bodies (Figure 4A). For ubiquitin, western blot analysis showed increased levels of both ubiquitinated proteins and monomeric ubiquitin (8.5 kDa) in patient muscle. Although the proteomics analysis identified proteins involved in the clearance of cellular aggregates, factors involved in effector stages of autophagy were not found. However, markers of autophagosome formation (LC3) and lysosomes (LAMP2) were colocalised with polyglucosan bodies by immunohistochemistry (Figure 4B). They had higher expression levels in patient muscle than in normal control muscle and comparable levels to that seen in a case of chloroquine myopathy with prominent activation of autophagocytosis (Figure 4C).

We further validated the MS results for four proteins with chaperone function (HSPB1, HSP70, CRYAB and RAD23B) and one structural protein (DES). For all five proteins, prominent immunolocalisation to polyglucosan was observed confirming the result from LMD-MS (Figure 5).

Immunohistochemical analysis was performed in two IBM patients with antibodies to proteins involved in glycogen metabolism and accumulated in polyglucosan bodies in PGBM1 patients. The IBM patients exhibited numerous rimmed vacuoles, which showed p62 (sequestosome-1)-positive protein aggregates, but there was no apparent accumulation of glycogen synthase, myophosphorylase, debranching enzyme, laforin or glycogenin-1 (Figure S2).

**DISCUSSION**

Polyglucosan bodies are the histopathological hallmark of PGBM1 and other diseases characterised by storage of polyglucosan, which consists of polyglucans and proteins forming dense and strongly PAS-positive aggregates. The exact composition of the aggregates in different polyglucosan storage disorders is not known, and only sparse

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**TABLE 3 (Continued)**

| Protein                        | UniProt | Gene   | MW   | UP | P1 | P2 | P3 | P4 | p   | PAR |
|-------------------------------|---------|--------|------|----|----|----|----|----|-----|-----|
| **Miscellaneous**             |         |        |      |    |    |    |    |    |     |     |
| Ferritin heavy chain          | P02794  | FTH1   | 21   | 1  | 4.6| 10.4| 3.6| -  | 0.03| 5.6 |
| Syntxin-binding protein 2     | Q15833  | STXB2  | 66   | 1  | 2.7| 3.2 | 3.1| -  | 0.00| 3.0 |
| Testis-specific serine/threonine-protein kinase 6 | Q9BXA6 | TSSK6 | 30   | 1  | 3.4| 3.2 | 1.9| 2.8| 0.00| 2.8 |
| Iroquois-class homeodomain protein IRX-4 | P78413 | IRX4  | 54   | 1  | 1.7| 2.0 | 1.7| 1.8| 0.00| 1.8 |
| Cytochrome b-c1 complex subunit 9 | Q9UDW1 | UQCR10| 7    | 1  | 0.9| 1.9 | 2.3| 1.8| 0.09| 1.7 |
| AMP deaminase 1               | P23109  | AMPD1 | 90   | 1  | 2.4| 1.7 | 1.5| 1.0| 0.09| 1.6 |
| Annexin A6                    | P08133  | ANXA6 | 76   | 3  | 1.4| 1.7 | 1.6| 1.5| 0.00| 1.6 |
| Reticulon-4                   | Q9NQC3  | RTN4  | 130  | 1  | 1.9| 1.4 | 1.3| -  | 0.07| 1.5 |
| 14-3-3 protein gamma          | P61981  | YWHAG | 28   | 3  | 1.3| 1.8 | 1.1| 1.4| 0.04| 1.4 |
| Phosphatidylethanolamine-binding protein 1 | P30086 | PEBP1 | 21   | 3  | 1.5| 1.1 | 1.5| 1.2| 0.04| 1.4 |
| WD repeat-containing protein 11 | Q9BZH6 | WDR11 | 137  | 1  | 1.5| 1.3 | 1.3| 1.2| 0.01| 1.3 |

**Note:** Listed are proteins with an adjusted p value (p) below 0.10 and an average protein accumulation ratio for all patients (PAR) above 1.20.

Abbreviations: MW, molecular weight in kDa; P1–P4, ratio of protein accumulation in polyglucosan from individual patients; UP, number of unique peptides.
information is available regarding the proteins that are part of the polyglucosan bodies. To get more insight into the pathobiology of polyglucosan body diseases, we performed quantitative MS analysis of the protein content of polyglucosan bodies in PGBM1, which is a disease with massive storage of polyglucosan aggregates in muscle that can readily be isolated by LMD for further analysis. This allowed us to define the main protein composition of polyglucosan bodies by direct coupling of the histologically defined PAS-positive polyglucosan aggregates to MS analysis using LMD.

The method was validated by immunohistochemical analysis of 17 selected proteins, which had been identified as accumulated by the MS analysis. The immunohistochemical validation included only one patient, but the proteins reported to be accumulated were increased in all patients as demonstrated by the LMD-MS method. These proteins showed a clear localization to the polyglucosan bodies by immunohistochemistry and were frequently depleted in the same fibres in regions outside the polyglucosan bodies. Notably, several accumulated proteins did not show a substantially altered overall expression in patient muscle, and therefore the accumulation of such proteins to polyglucosan bodies probably reflects a redistribution. However, some other proteins appeared to be more abundant as revealed by western blot analysis.

| Protein | UniProt | Gene | MW | UP  | P1  | P2  | P3  | P4  | P  | PAR |
|---------|---------|------|-----|-----|-----|-----|-----|-----|----|-----|
| PDZ and LIM domain protein 5 | Q96HC4 | PDLM5 | 64  | 2   | 0.4 | 0.4 | 0.4 | 0.4 | 0.00 | 0.4 |
| Myozin-2 | Q9NPC6 | MYOZ2 | 30  | 1   | 0.9 | 0.3 | 0.3 | 0.3 | 0.04 | 0.4 |
| Myotilin | Q9UBF9 | MYOT | 55  | 12  | 0.5 | 0.5 | 0.5 | 0.6 | 0.00 | 0.5 |
| Alpha-actinin-2 | P35609 | ACTN2 | 104 | 25  | 0.4 | 0.5 | 0.6 | 0.6 | 0.01 | 0.5 |
| Myozin-1 | Q9NP98 | MYOZ1 | 32  | 6   | 0.3 | 0.4 | 0.6 | 0.7 | 0.02 | 0.5 |
| Tropomyosin beta chain | P07951 | TPM2 | 33  | 9   | 0.4 | 0.4 | 0.6 | 0.6 | 0.01 | 0.5 |
| Myomesin-2 | P54296 | MYOM2 | 165 | 18  | 0.5 | 0.5 | 0.6 | 0.8 | 0.02 | 0.5 |

TABLE 4: Proteins with reduced quantity in polyglucosan from PGBM1 patient muscle

Note: Listed are proteins with an adjusted p value (p) below 0.10 and an average protein accumulation ratio for all patients (PAR) below 0.66.
Abbreviations: MW, molecular weight in kDa; P1–P4, ratio of protein accumulation in polyglucosan from individual patients; UP, number of unique peptides.
A major functional group of proteins accumulated in polyglucosan in PGBM1 muscle were key enzymes involved in glycogen synthesis and degradation. Several of these proteins are known to physically associate with glycogen. Glycogen synthase has multiple glycogen-binding sites, which are necessary for efficient catalytic activity.\(^\text{19}\) Glycogenin-1 is an essential primer for glycogen synthesis and is thus a part of glycogen and also binds to glycogen synthase.\(^\text{20,21}\) Laforin has a carbohydrate-binding domain of a very specific type (carbohydrate-binding module 20, CBM20), which is thought to bind a laforin-malin complex to elongating long-branched glucans in glycogen to inhibit further elongation and precipitation.\(^\text{9}\) Glycogen phosphorylase\(^\text{22}\) and debranching enzyme\(^\text{23}\) also exhibit glycogen-binding sites. Branching enzyme, which also contains a carbohydrate-binding domain,\(^\text{24}\) was not detected in the MS analysis although branching enzyme protein levels in PGBM1 patients were similar to controls when analysed by western blot (not shown). However, branching enzyme does not seem to form stable interactions with glycogen particles prepared from muscle and thus may not accumulate in polyglucosan.\(^\text{25}\) The enzymes associated with glycogen metabolism that were identified in the polyglucosan bodies largely overlap with those found in normal glycogen particles isolated from mouse and rat liver and mouse 3T3-L1 adipocytes.\(^\text{26,27}\) Therefore, accumulation of several key enzymes of glycogen metabolism in polyglucosan bodies may be due to interactions with the carbohydrate and protein components.
of polyglucosan, through carbohydrate-binding modules and protein–protein interactions.

It was not possible to dissect aggregates and control tissue from the same muscle fibres due to the dispersed distribution of polyglucosan in affected fibres. Control tissue was therefore collected from adjacent but separate fibres with normal morphology. In these fibres, proteins were not reduced in the cytoplasm by sequestration to polyglucosan. Thus, the true accumulation ratios in affected fibres were probably underestimated (e.g., GYS1 in Figure 3).

Sequestration of the glycogen metabolism machinery components into polyglucosan bodies, as indicated by LMD-MS and verified by immunohistochemistry, results in depletion of glycogen in other parts of afflicted fibres, as demonstrated by PAS histochemistry. To our knowledge, this is the first study to comprehensively describe the sequestration of core glycogen metabolic enzymes to polyglucosan.
bodies causing glycogen depletion. This metabolic deficiency in a large proportion of the muscle fibres most likely results in impairment of muscle function similar to what is found in glycogen synthase deficiency. Inactivation of the \textit{GYS1} gene, resulting in glycogen synthase deficiency and absence of glycogen as a source for rapidly degradable glucose, is associated with severe muscle weakness and profound exercise intolerance.²⁸

The largest subset of proteins identified in polyglucosan bodies from PGBM1 patients is associated with various abnormalities of protein quality control. A number of chaperones (heat shock proteins) were accumulated in polyglucosan indicating that protein misfolding and aggregation occur at high levels in diseased muscle fibres. Several of the identified chaperones have previously been described in muscle, mainly in relation to maintenance of the contractile apparatus.²⁹,³⁰

The substrate for the chaperones is unknown, but the abnormal accumulation of proteins related to glycogen metabolism may result in misfolding and aggregation of such proteins and drive the recruitment of the chaperone system. Several of the chaperones that we identified in polyglucosan bodies (HSP90, HSPA1A, HSPA8) are also involved in clearance of misfolded or damaged proteins by interaction with the systems for protein degradation described below.³⁰

Our results further demonstrate a prominent localisation to polyglucosan of factors involved in degradation of proteins and protein aggregates through the ubiquitin-proteasome and autophagy-lysosome systems. Ubiquitin, regarded as the pivotal molecular clue for protein degradation, was highly accumulated in polyglucosan and conjugated to various proteins in PGBM1 muscle. Alongside ubiquitin, we found UCHL1, which is involved in processing of newly synthesised ubiquitin and KILH41 and GPS1 that control the ligation of ubiquitin to substrate proteins. Furthermore, sequestosome-1 (p62), VCP, NSFL1 and RAD23B, which are known to bind ubiquitin and function as bridging factors between ubiquitinylated proteins and the proteasome or autophagy degradation pathways, were identified by our LMD-MS approach. As these ubiquitin-binding proteins may interact with subunits of the proteasome as well as autophagy pathways, their presence does not indicate which route of degradation is active.³¹ We identified several key subunits of the 20S catalytic core and 26S regulatory domain of the proteasome by LMD-MS and further verified the accumulation of proteasomes in polyglucosan by immunohistochemistry. Our results therefore indicate that proteasomes are recruited to polyglucosan bodies. Proteins involved in autophagy after substrate recognition were not identified by our LMD-MS analysis. However, LC3 and LAMP2 were detected in polyglucosan by immunohistochemistry and found to be upregulated by western blot analysis indicating the presence of both autophagosomes and lysosomes. Activation of the cellular control systems may be similar in different polyglucosan storage diseases since immunohistochemical studies have revealed ubiquitin and p62 in polyglucosan in Lafora disease, glycogenin-1 deficiency and branching enzyme deficiency.⁶,³²,³³

The accumulation of proteins involved with various aspects of cellular quality control in polyglucosan bodies points to insufficient function of the ubiquitin-proteasome and autophagy systems. The RBCK1-containing LUBAC complex and otulin have been demonstrated to control assembly of autophagosomes by regulating Atg13 stability through linear ubiquitination and de-ubiquitylation, respectively.³⁴ Although RBCK1 deficiency thus may lead to impaired autophagy, it is unlikely that defective autophagy is the primary cause of PGBM1 pathology, and it should be noted that myopathies with primary defects in autophagy generally do not feature polyglucosan bodies.³⁵ However, impaired autophagy may play a part in the pathobiology as has been suggested for glycogen storage disease II and III.³⁶,³⁷ In addition, RBCK1 is an E3 ubiquitin ligase, and there may be a so far not identified substrate for RBCK1, which is dysregulated and
essential for the formation of polyglucosan. Given the morphological and biochemical similarities of polyglucosan bodies in PGBM1 to other glycogen storage diseases with polyglucosan storage, it is likely that deficiency of RBCK1 affects molecular processes central for glycogen metabolism. In this context, it is of interest that laforin and glycogenin-1, which are also associated with polyglucosan storage when they are inactivated by mutations, showed the highest accumulation in polyglucosan in RBCK1 deficient patients.

The formation of polyglucosan bodies in inherited diseases has been attributed to faulty glycogen synthesis and the production of elongated glucan chains with lack of branching leading to insoluble molecules and aggregation. This may be caused by an imbalance of the enzymatic activities of glycogen related proteins, for instance, due to hypomorphic mutations in GBE1 encoding branching enzyme,38,39 or overexpression of glycogen synthase.40,41 In Lafora disease, a central quality control mechanism, prohibiting extensive glucan chain elongation in glycogen, is disabled by mutations of either laforin or malin.9

Our analysis of the protein content of PGBM1 polyglucosan highlights cellular processes involved in protein aggregation and clearance and thereby points to similarities between polyglucosan and protein aggregates found in various muscle and neurodegenerative diseases. Several of the proteins accumulated in polyglucosan are present in protein aggregate myopathies like myotilinopathy and filaminopathy, for example, HSPB1, alpha-crystallin B chain, p62, VCP and the muscle-specific intermediate filament desmin.37,42 Protein components found in polyglucosan of patients with RBCK1 deficiency are also seen in protein aggregates of frontotemporal dementia and amyotrophic lateral sclerosis, for example, UCHL-1 and RAD23B.43,44 These similarities between polyglucosan and well-established protein aggregate diseases indicate that protein aggregation is a contributing factor for the establishment and persistence of polyglucosan bodies. Future treatment strategies aiming at reducing the polyglucosan load will likely need to address aspects of protein aggregation in parallel with the glucan component, and this may also be important in other glycogen storage disorders such as Lafora disease and branching enzyme deficiency.

CONCLUSIONS

Our results demonstrate redistribution of key enzymes of glycogen metabolism to the polyglucosan bodies, explaining the glycogen depletion in numerous fibres resulting in muscle function impairment with weakness and wasting. The accumulation of components of the protein quality control systems and other proteins frequently found in protein aggregate disorders indicate that protein aggregation may be an essential part of the pathobiology of polyglucosan storage.

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CONFLICT OF INTEREST

None of the other authors report any conflict of interest.

ETHICS STATEMENT

The study was approved by the regional ethical review board in Gothenburg, Sweden. The investigated individuals gave their informed consent.

AUTHOR CONTRIBUTIONS

CT and AO designed the study and experiments. CT performed experiments. CT and AO interpreted data. AO, EM, AJ, MR, OK and CL assessed patients and clinical parameters. AO, EM, AJ, MR and OK provided samples. CT and AO wrote the manuscript. All authors commented and approved the manuscript.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article.

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