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Novel method for detection of glycogen in cells

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Abstract

Glycogen, a branched polymer of glucose, functions as an energy reserve in many living organisms. Abnormalities in glycogen metabolism, usually excessive accumulation, can be caused genetically, most often through mutation of the enzymes directly involved in synthesis and degradation of the polymer leading to a variety of glycogen storage diseases (GSDs). Microscopic visualization of glycogen deposits in cells and tissues is important for the study of normal glycogen metabolism as well as diagnosis of GSDs. Here, we describe a method for the detection of glycogen using a renewable, recombinant protein which contains the carbohydrate-binding module (CBM) from starch-binding domain containing protein 1 (Stbd1). We generated a fusion protein containing glutathione S-transferase, a cMyc eptitope and the **S**tbd1**C**BM (GYSC) for use as a glycogen-binding probe, which can be detected with secondary antibodies against glutathione S-transferase or cMyc. By enzyme-linked immunosorbent assay, we demonstrate that GYSC binds glycogen and two other polymers of glucose, amylopectin and amylose. Immunofluorescence staining of cultured cells indicate a GYSC-specific signal that is co-localized with signals obtained with anti-glycogen or anti-glycogen synthase antibodies. GYSC-positive staining inside of lysosomes is observed in individual muscle fibers isolated from mice deficient in lysosomal enzyme acid alpha-glucosidase, a well-characterized model of GSD II (Pompe disease). Co-localized GYSC and glycogen signals are also found in muscle fibers isolated from mice deficient in malin, a model for Lafora disease. These data indicate that GYSC is a novel probe that can be used to study glycogen metabolism under normal and pathological conditions.

Key words: CBM20, glycogen, immunofluorescence, Lafora disease, Pompe disease

Introduction

Glycogen is a branched polymer of glucose and is the primary carbohydrate storage form in animals (Agius 2008; Roach et al. 2012). Glycogen synthesis and degradation are tightly controlled by complex regulatory mechanisms (Agius 2008; Roach et al. 2012; Adeva-Andany et al. 2016). Disturbances in this regulation or in the metabolic enzymes themselves can result in aberrant glycogen stores, resulting in a glycogenosis or glycogen storage disease (GSD) (Chen and Burchell 1995; DiMauro and Lamperti 2001; DiMauro and Spiegel 2011; Oldfors and DiMauro 2013). These are monogenic, congenital disorders in which a genetic defect results in abnormal amounts and/or structure of glycogen. The first examples involved mutations linked directly to glycogen metabolizing enzymes, such as GSD II (Pompe disease) in which the lysosomal α-glucosidase is defective (Reuser et al. 1995; Hirschhorn and Reuser 2000; Raben et al. 2002). Histochemical staining of glycogen is therefore important diagnostically, the most commonly used method being periodic acid Schiff (PAS) staining that visualizes molecules with a high percentage of carbohydrate content (McManus 1946; Hotchkiss 1948). One disadvantage of PAS staining for the detection of glycogen in tissues is lack of specificity. Besides glycogen, PAS staining detects other carbohydrate containing molecules including glycoproteins,

glycolipids and mucins (McManus 1946; Hashimoto et al. 1965; Dahr et al. 1976). Indeed, it is used in diagnosis of conditions such as Paget's disease of the breast (Crignis et al. 2013), adenocarcinoma (Warnock et al. 1988), glomerular diseases in the kidney (Nakatani et al. 2004) and others. Another disadvantage of PAS staining is its general incompatibility with immunofluorescence staining techniques, precluding analysis of the co-localizaton of glycogen with protein molecules, an approach that could be useful for elucidation of molecular mechanisms underlying various GSDs. An antibody that recognizes glycogen (IV58B6) has been quite widely used in studies of cells and tissues. Baba (1993) used mandibular condylar cartilage as an antigen to produce monoclonal antibodies in mouse that were subsequently found to recognize glycogen although cross-reactivity with other molecules cannot be rigorously excluded. We sought to generate a new renewable probe to specifically detect glycogen in cultured cells or tissues. The probe contains the carbohydrate-binding module (CBM) from human starch-binding domain containing protein 1 (Stbd1) fused to GST and c-myc tags. Human Stbd1 is a 39 kDa protein that consists of a highly conserved N-terminal hydrophobic sequence that targets the protein to membranes, a disordered central region and a C-terminal CBM20, that has been shown to bind to glycogen (Jiang et al. 2010). We validate that the resulting probe can bind to glycogen in vitro and that it can be used for immunofluorescent detection of glycogen in mammalian cells and in individual muscle fibers from mouse models of Lafora and Pompe diseases. Furthermore, this probe is compatible for protein co-localization studies.

Results

Design, expression and purification of a glycogen-binding probe

Previous studies had demonstrated that Stbd1 binds to glycogen in vitro and co-localized with glycogen in mammalian cells (Jiang et al. 2010). To generate a probe capable of detecting glycogen, we designed a bacterial expression plasmid that would encode a fusion protein containing glutathione S-transferase (GST), a cMyc-tag and a carbohydrate-binding module corresponding to residues 261–358 of human Stbd1 (Figure 1 A). The resulting protein, named GYSC, was expressed in *Escherichia coli* strain BL21, purified by absorption on glutathione-agarose beads, eluted with glutathione and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified fractions contained a predominant band with Mr ~ 39 kDa (Figure 1B).

Carbohydrate-binding specificities of GYSC and anti-glycogen antibody

In order to characterize the carbohydrate-specificity of GYSC, we used glycogen and two other glycogen-like polymers, amylopectin and amylose, which differ from glycogen in their degree of branching. Mammalian glycogen is branched on average at 1 in ~13 glucose residues, amylopectin 1 in 20–25 and amylose is scarcely branched at all (Roach et al. 2012; Roach and Zeeman 2016). Binding analysis was performed by enzyme-linked immunosorbent assay (ELISA). GYSC bound to all three polymers of glucose with slightly higher affinity to amylopectin and lower affinity to amylose, as compared to glycogen (Figure 2 A). Half maximal effective concentrations (EC_{50}) of glycogen, amylopectin and amylose for GYSC binding were 0.18, 0.07 and 0.57 µg/mL, respectively. We also analyzed binding of the Baba antibody (Baba 1993) to the same

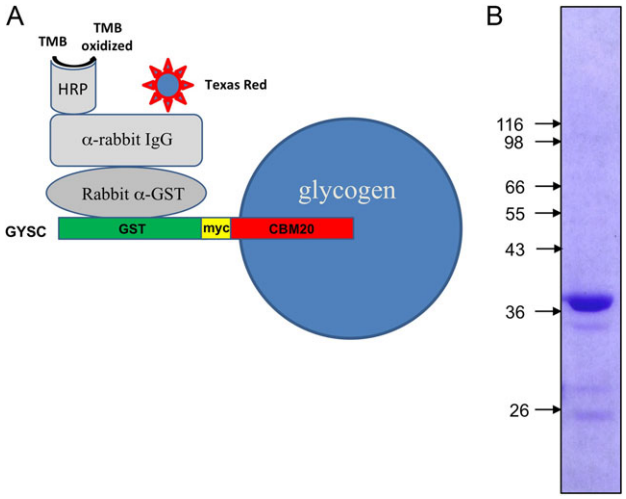

Fig. 1. Scheme for experimental procedures with GYSC and analysis of purified GYSC by SDS-PAGE. In (**A**), a fusion protein GYSC containing the CBM20 domain from human Stbd1 with attached two epitope tags, cMyc (myc) and GST is shown. The CBM20 domain serves for binding of GYSC to glycogen, whereas GST-tag is used for detection of glycogen-bound GYSC by either ELISA or immunofluorescent microscopy. In both methods, the GYSC–glycogen complex is incubated first with rabbit anti-GST antibody and then with anti-rabbit secondary antibody conjugated with HRP (ELISA) or fluorophore (Texas Red is shown as an example). In (**B**), Coomassie blue stained SDS-PAGE gel of purified GYSC is shown. Numbers on the left indicate migration of molecular mass markers (in kDa). GYSC, a fusion protein composed of glutathione S-transferase, a cMyc eptitope and the Stbd1CBM; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBM, carbohydrate-binding module; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase.This figure is available in black and white in print and in color at *Glycobiology* online.

polysaccharides. As expected, this antibody bound glycogen (EC_{50} = 0.92 µg/mL) but it did not recognize amylopectin or amylose (Figure 2B), consistent with a previous report that the epitopes recognized by Baba antibody correspond to densely branched regions of glycogen (Nakamura-Tsuruta et al. 2012). Therefore, GYSC is less sensitive to the branching pattern of polysaccharides than the Baba antibody. Data on the glucan binding characteristics of the Stbd1 CBM20 domain are sparse but the results are consistent with the known substrate-binding specificity of CBM20 domains from other proteins. Previous studies demonstrated that CBM20 has optimal affinity towards linear maltooligosaccharides of approximately DP7 composed of solely α-(1–4) linkages and it does not bind oligosaccharides smaller than maltopentaose (Sorimachi et al. 1997; Giardina et al. 2001; Tanackovic et al. 2016)

Detection of glycogen by GYSC in mammalian cells

To test whether GYSC can detect glycogen in mammalian cells, we first utilized Rat-1 fibroblasts that stably overexpress glycogen synthase (WT-4 line) (Skurat et al. 2000) and have a 5-fold increase in glycogen accumulation over control cells (not shown). In this cell model, large glycogen deposits were localized predominantly in a perinuclear area as detected by immunofluorescence microscopy using the anti-glycogen antibody (Figure 3B). With GYSC as a glycogen probe (Figure 3 A), detection of GYSC with anti-GST antibodies for the most part overlapped the staining obtained with the anti-glycogen antibody (Figure 3 C). Glycogen synthase is closely associated with glycogen in cells (Prats et al. 2009) and so we also

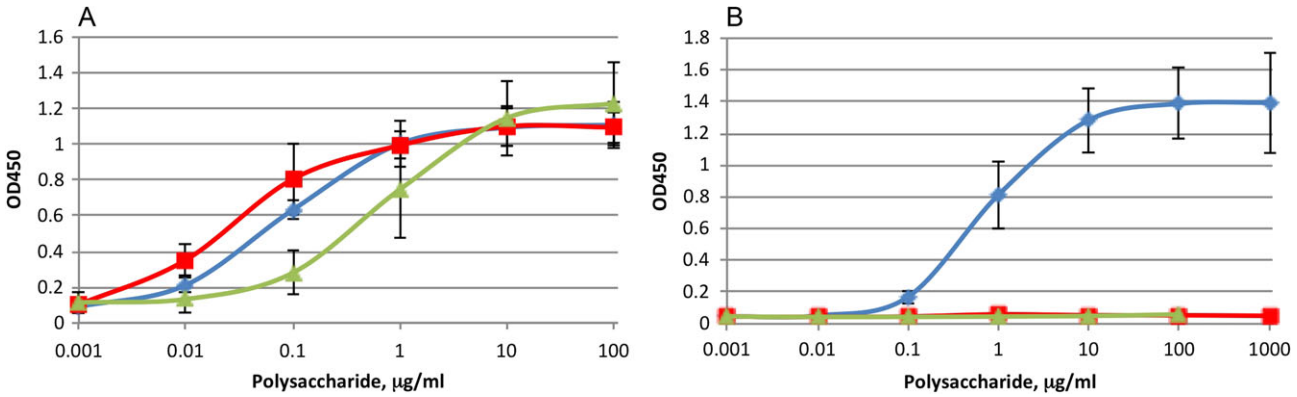

Fig. 2. Binding analysis of GYSC or glycogen antibody to polysaccharides. Binding of GYSC (**A**) or anti-glycogen antibody (**B**) to glycogen (diamonds), amylopectin (squares) or amylose (triangles) was analyzed by ELISA as described in the "Materials amd methods" section. Error bars indicate SD for four independent experiments. This figure is available in black and white in print and in color at *Glycobiology* online.

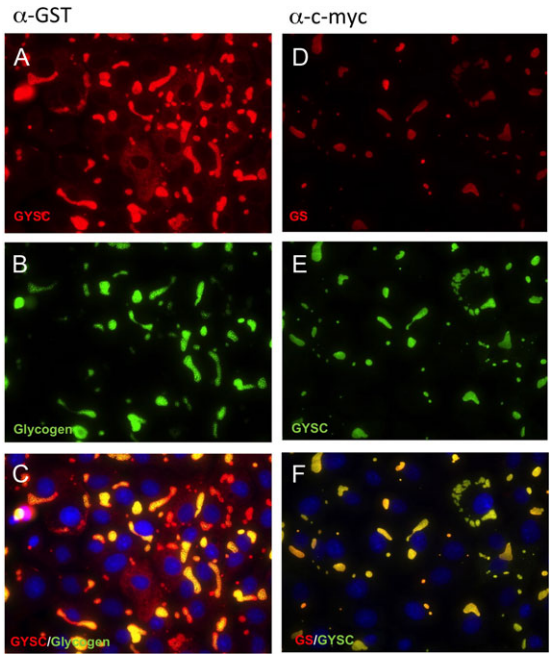

Fig. 3. Co-localization of GYSC, glycogen and glycogen synthase (GS). Rat-1 fibroblasts stably expressing wild type glycogen synthase (cell line WT-4) were immunostained with GYSC and anti-glycogen antibody (**A–C**) or GYSC and anti-glycogen synthase antibody (**D–F**). To detect GYSC localization either anti-GST antibody (A–C) or anti-C-myc antibody (D–F) were used. The images were merged (C and F). Nuclei were stained with Hoechst. This figure is available in black and white in print and in color at *Glycobiology* online.

compared the localization of GYSC and glycogen synthase (Figure 3D–F), in this experiment visualizing GYSC with anti-c-myc antibodies. The results indicate a strong co-localization of GYSC and glycogen synthase (Figure 3 F). In addition, there was a similar pattern of GYSC staining whether it was detected by α-GST or α-cMyc antibodies, providing further validation of the GYSC localization.

In a second series of experiments, we tested whether GYSC could detect endogenous glycogen in wild type Rat-1 fibroblasts and monitor its response to changes in nutritional status. In cells grown under standard conditions with 25 mM glucose, immunofluorescence microscopy revealed GYSC- and anti-glycogen antibody-positive staining in small punctate structures scattered throughout the cytoplasm (not shown but similar to the pattern in Figure 4D–F). Starvation of cells in medium lacking glucose led to disappearance of this staining by either GYSC or anti-glycogen antibody (Figure 4 A–C). Refeeding of starved cells with medium containing 25 mM glucose for 6 h caused re-appearance of the punctate staining. Signals from GYSC and anti-glycogen antibody demonstrated near complete co-localization (Figure 4 F). As further proof that the observed immunofluorescent labeling is due to glycogen, we treated re-fed fixed cells with glycogen-degrading enzymes prior to incubation with GYSC or anti-glycogen antibody. Treatment with either diastase (Figure 4 G) or amyloglucosidase (Figure 4 H) completely eliminated the labeling, confirming that glycogen is necessary for GYSC or the Baba antibody to generate a fluorescence signal. GYSC- and anti-glycogen antibody-staining correlated with enzymatic measurements of glycogen in Rat-1 fibroblasts. Indeed, glucose starvation caused almost 20-fold decrease in glycogen level and refeeding for 6 h almost completely restored it (Figure 4I). The punctate pattern of glycogen distribution was also observed in other mammalian cells (not shown).

In a further control, we sought to confirm that the signal detected with GYSC required the presence the Stbd1 CBM20 domain. Therefore, with re-fed Rat-1 fibroblasts, we compared GYSC and purified GST protein as probes for glycogen (Figure 5 A,D). GYSC gave the expected punctate pattern (as in Figure 4D), whereas no signal was detected with GST alone (Figure 5D), indicating that the GYSC signal is specific. The cells were also probed with α-glycogen synthase antibodies as a mean to localize glycogen resulting in the normal punctate distribution (Figure 5B,E). Thus, the normal glycogen pattern was present even when GST failed to reveal any signal.

GYSC detection of glycogen in muscle of mouse models of GSDs

Aberrant glycogen structure and deposition are associated with the various GSDs. To determine whether GYSC could be used for analysis of abnormal glycogen stores, we utilized two mouse models for GSDs, Lafora and Pompe disease. In Lafora disease, mutations of either the *Epm2a* or *Epm2b* genes lead to the formation of insoluble deposits of abnormal glycogen that is a major constituent of Lafora bodies, a hallmark of the disease in humans and mice (Collins et al. 1968; Carpenter and Karpati 1981; Ganesh et al. 2002; DePaoli-Roach et al. 2010). The glycogen is poorly branched and excessively

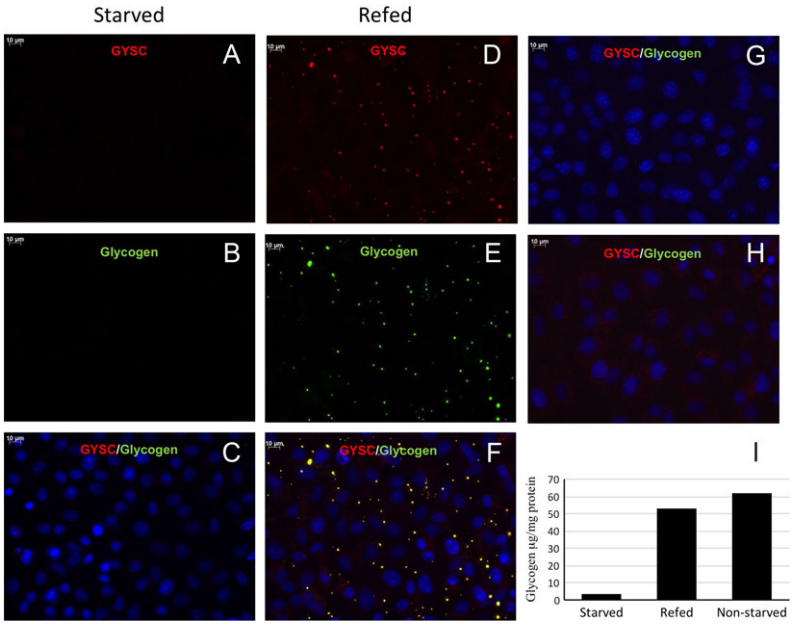

Fig. 4. Detection of endogenous glycogen in Rat-1 fibroblasts with GYSC. Rat-1 fibroblasts were starved in the medium lacking glucose for 48 h. Cells were then left either in the no glucose medium (**A–C**) or incubated in the medium containing 25 mM glucose for 6 h (**D–H**). To digest glycogen, fixed cells were treated with diastase (G) or amyloglucosidase (H). Glycogen was visualized with GYSC or anti-glycogen antibody. GYSC was detected with anti-GST antibody. The images were merged (C, F, G and H). Nuclei were stained with Hoechst. Glycogen content in starved, re-fed and nonstarved Rat-1 fibroblast from one representative experiment is shown (**I**). This figure is available in black and white in print and in color at *Glycobiology* online.

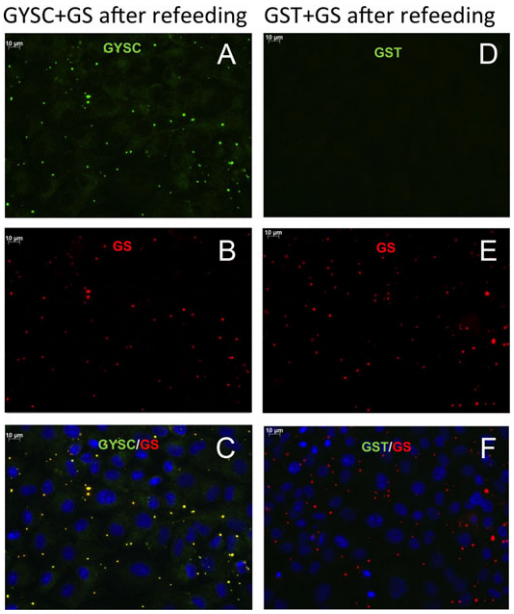

Fig. 5. Co-localization of GYSC and GST with glycogen synthase (GS). Rat-1 fibroblasts were starved and refed as described in the legend to Figure 4. Fixed cells were incubated with GYSC (**A–C**) and GST (**D–F**) followed by incubation with anti-GST and anti-glycogen synthase antibody. Nuclei were stained with Hoechst. This figure is available in black and white in print and in color at *Glycobiology* online.

phosphorylated (Tagliabracci et al. 2007, 2008). Lafora bodies are found in many tissues, including skeletal muscle, heart, skin and neurons, the last likely being causative of the associated epilepsy and neurodegeneration (Van Heycop Ten Ham 1975; Delgado-Escueta et al. 2001). Immunofluorescence imaging of individual muscle fibers from *Epm2b*$^{-/-}$ mice revealed multiple deposits stained with both GYSC and anti-glycogen antibody (Figure 6). These data indicate that GYSC stains the abnormal glycogen-like material in Lafora bodies.

In Pompe disease, lysosomal glycogen disposal is defective due to mutation of the lysosomal α-glucosidase (acid maltase; GAA) resulting in massive accumulation of glycogen in the lysosomes, disrupting autophagic trafficking and lysosome function generally (Hirschhorn and Reuser 2000; Raben et al. 2008). Skeletal muscle and heart are typically the organs most affected. Analysis of muscle fibers from a mouse model of Pompe disease by confocal microscopy revealed a different pattern of GYSC staining (Figure 7) compared with the *Epm2b*$^{-/-}$ fibers. GYSC-positive structures of irregular shape were scattered throughout the depth and length of the muscle fiber. Staining with antibody against an endosomal/lysosomal marker, LAMP1, demonstrated markedly enlarged lysosomes, a characteristic feature of Pompe disease. Staining using both GYSC and LAMP1 antibody indicated that GYSC-positive material is localized inside the lysosomes (Figure 7). No GYSC-positive staining was seen inside the lysosomes in muscle fibers isolated from wild type mice (not shown).

Discussion

Glycogen particles in muscle have average dimensions of ~25 nm (Shearer and Graham 2004) and so visualization at a true molecular level requires electron microscopy. However, detection of glycogen in cells and tissues has long been achieved by histochemical methods, the most common of which is PAS staining whereby oxidation of hydroxyls in glucose residues by periodic acid is followed by reaction of the resulting aldehydes with Schiff reagent to generate a purple or magenta color (Pearse 1961). The stain detects any structure with a high content of carbohydrate, including glycogen. PAS-stained sections of liver cells, for example, even at relatively low

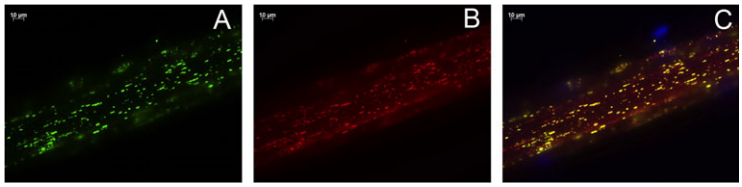

Fig. 6. Detection of Lafora bodies in single psoas fiber from *Epm2b*$^{-/-}$ mice. The immunofluorescence analysis was performed with anti-glycogen antibodies (**A**) and GYSC (**B**). The images were merged (**C**). Nuclei were stained with Hoechst. This figure is available in black and white in print and in color at *Glycobiology* online.

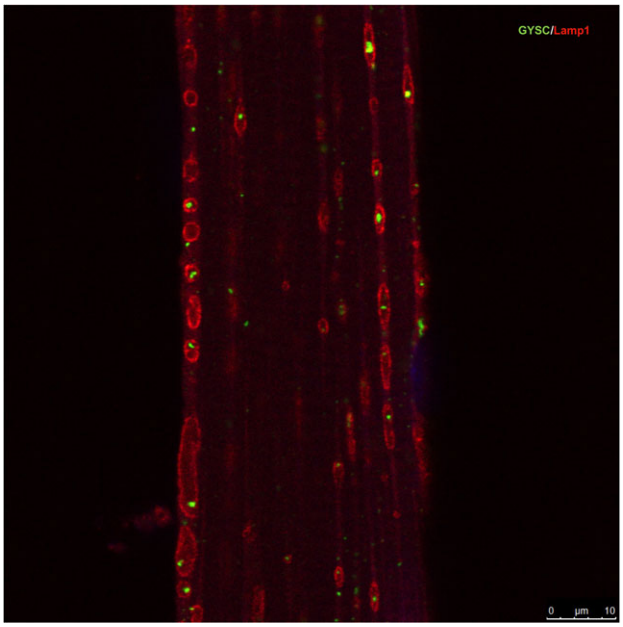

Fig. 7. Localization of glycogen within lysosomes in single anterior tibialis fiber from GAA$^{-/-}$ mice. Confocal image of single muscle fiber stained with GYSC and antibody against lysosomal membrane protein Lamp1. The images were merged. Nuclei were stained with Hoechst. A representative Z-stack image is shown. This figure is available in black and white in print and in color at *Glycobiology* online.

magnification, readily indicate the level of glycogen stored in the hepatocytes via the intensity of the staining (Hammad et al. 1982). PAS staining has also been used extensively to assist in the diagnosis of a variety of GSDs (Sato 2002; Kishnani et al. 2010; Escobar et al. 2012).

Glycogen is generally associated with the proteins involved in its metabolism and so immunochemical detection of these proteins might provide a surrogate to identify glycogen localization. Glycogen synthase, for example, is often tightly associated with glycogen and commercial antibodies are available. However, under some physiological and pathological conditions, glycogen and glycogen synthase are not co-localized. For example, glycogen synthase is present only in the cytosol in several mammalian cell lines under normal conditions but prolonged exposure of cells in glucose-free medium or activation of glycogen phosphorylase causes glycogen depletion and translocation of glycogen synthase from the cytosol to the nucleus (Cid et al. 2005). Another example of lack of co-localization of glycogen and glycogen synthase is in Pompe disease that is caused by deficiency of the lysosomal glycogen-degrading enzyme, acid α-glucosidase. Lysosomal protein-degrading enzymes are unaffected and thus glycogen accumulation in lysosomes could not be reliably followed by glycogen synthase.

Finally, the amount of glycogen synthase in tissues does not always correlate with the amount of glycogen. For example, starvation causes dramatic reduction of glycogen in liver and adipose tissue without substantial alteration in glycogen synthase expression (Gannon and Nuttall 1997; Carmean et al. 2016). Therefore, comparative analysis of glycogen levels in different metabolic states cannot be performed using anti-glycogen synthase antibodies.

Antibodies have been developed that purport to directly recognize glycogen (Baba 1993; Nakamura-Tsuruta et al. 2012). These antibodies are not commercially available and we decided to develop a more directed and renewable probe for glycogen, GYSC, based on the properties of the CBM domain of the protein Stbd1. We had previously proposed that Stbd1 is involved in glycogen metabolism and, even though its precise role remains unclear, we have shown that Stbd1 binds to glycogen, in vitro and in cells (Jiang et al. 2010). In the present study, we believe that we have validated the effectiveness of GYSC as a novel probe to detect glycogen in cultured cells and isolated mouse muscle fibers.

Part of the validation was to compare GYSC with the Baba anti-glycogen antibody. In vitro, both recognized mammalian glycogen as judged by ELISA. GYSC also bound to the less branched plant polysaccharides amylopectin and amylose whereas the Baba antibody did not, at least under the conditions used. In fact, the inability of the Baba antibody to bind less branched polymers of glucose has been described in previous studies by van de Weerd et al. (2015) who proposed that the result was explained by the structure of the antibody. The Baba antibody is a pentameric IgM immunoglobulin that contains 10 binding sites capable of multivalent interactions with carbohydrate ligands. van de Weerd et al. (2015) suggested that high affinity binding requires the antibody to interact with multiple α-1,4-linked linear maltotriose epitopes, potentially straddling multiple polyglucose chains. Therefore, more densely branched glucose polymers like glycogen may provide more effective epitopes for each molecule of the multivalent antibody when compared with less branched polymers. In contrast, the smaller GYSC is able to bind glucose polymers such as amylose that are almost devoid of branches, possibly indicating that the binding "epitope" is a short segment of polyglucose. However, when used to detect glycogen in cultured cells, both the Baba antibody and GYSC gave similar and consistent results, with strong co-localization of the respective signals. As a more stringent test, we assessed the ability of GYSC to visualize glycogen deposits in muscle fibers from two animal models of GSDs, Pompe disease and Lafora disease.

We were able to demonstrate co-localization of glycogen and lysosomes in muscle fibers from Pompe mice (Figure 7), to our knowledge the first visualization of glycogen within the lysosome by immunofluorescent staining. Interestingly, this was the one instance

where the behaviors of GYSC and the Baba antibody differed, the anti-glycogen antibody failing to detect glycogen inside the lysosomes. Possibly, the lysosomal membrane is not permeable to the anti-glycogen antibody under the conditions used for preparation of muscle fibers. The large size of the IgM molecule (970 kDa) may make it be too large to cross the lysosomal membrane whereas GYSC is much smaller, with molecular mass of a monomer ~39 kDa (Figure 1B).

GYSC also detected glycogen deposits in muscle fibers from a mouse model of Lafora disease, *Epm2b*$^{-/-}$ mice (Figure 6). In this case, both GYSC and the Baba antibody gave signals that were essentially coincident. One could expect that the anti-glycogen antibody would be ineffective in recognizing the glycogen in the *Epm2b*$^{-/-}$ mice since it is poorly branched, especially in Lafora bodies. However, it is only a subset of the Lafora glycogen that is abnormal and in addition, the aggregates of glycogen molecules that become insoluble will likely carry along normally branched glycogen.

In conclusion, we have developed and validated a novel probe for glycogen that may have utility for the study of glycogen in cells and which may help advance understanding of the abnormal glycogen metabolism associated with GSDs.

Materials and methods

Antibodies

Rabbit anti-GST antibody and mouse monoclonal anti-glycogen antibody IV58B6 were kindly provided by Dr Clark Wells (Indiana University School of Medicine, Indianapolis, IN) and Dr O. Baba (Tokyo Medical and Dental University, Tokyo, Japan), respectively. Other primary antibodies include anti-glycogen synthase (AHO1412, Invitrogen, Camarillo, CA and NB110-57010, Novus Biologicals, Littleton, CO), anti-Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Lamp1 (1D4B, Developmental Studies Hybridoma Bank, Iowa City, IA).

Plasmid construction and protein purification

To generate pGEX-myc-Stbd1CBM, a DNA fragment encoding residues 261–358 of human Stbd1 was amplified by PCR using Stbd1 cDNA as template and two primers, one of which additionally contained sequence encoding a cMyc epitope tag (EQKLISEEDL). The resulting DNA fragment was subcloned into pGEX-4T1 to generate a vector that would encode a fusion protein containing GST–cMyc–Stbd1CBM, which we named GYSC. The plasmid was used to transform *E. coli* cell BL21/DE3. Transformants were isolated, induced with IPTG and the recombinant protein, GYSC, was purified over glutathione agarose. Bound GYSC was eluted with glutathione elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0), fractions were collected and analyzed by SDS-PAGE followed by Coomassie blue staining. Fractions with highest concentration of GYSC were pooled, frozen in liquid nitrogen and stored at −80°C.

Enzyme-linked immunosorbent assay

Rabbit liver glycogen (G-8876, Sigma-Aldrich, St Louis, MO) and potato amylopectin (A8515, Sigma-Aldrich) were dissolved in phosphate-buffered saline (PBS) to the final concentration of 1 mg/mL. Corn amylose (A-7043, Sigma-Aldrich) was dissolved in 1 M NaOH at 50 mg/mL and then diluted in PBS to a final concentration of 0.1 mg/mL. Serial dilutions of the polysaccharides in PBS were prepared and used to coat the wells of microtiter plates (3590, Costar, Cambridge, MA) for 2 h at 37°C. After coating, blocking buffer (BB), PBS containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 was added, and the plate was incubated for 2 h at room temperature. For determination of GYSC binding to polysaccharides, purified GYSC was diluted in BB to a final concentration of 0.55 µg/mL, added to the wells and incubated for 16 h at 4°C. To detect bound GYSC, rabbit anti-GST antibody diluted 1:1000 was added to the wells and incubated for 2 h at room temperature followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (31460, Thermo Fisher Scientific, Rockford, IL) diluted 1:30,000. The bound antibody was detected by adding 0.1 mL TMB reagent (Ultra TMB-ELISA, Thermo Fisher Scientific) followed by termination of the reaction by 0.1 mL 2 M H_2SO_4 and measurement of absorbance at 450 nm. Between each step, the wells were washed three times with buffer consisting of PBS and 0.1% Triton X-100.

Binding of anti-glycogen antibody to polysaccharides was measured by a similar procedure. After blocking, mouse anti-glycogen antibody was added to the wells and incubated for 16 h at 4°C. As a secondary antibody, HRP-labeled goat anti-mouse IgG+IgM antibody (31444, Thermo Fisher Scientific) was used.

Cell culture, glucose starvation and refeeding

Rat-1 fibroblasts were grown on chamber slides (80826, Ibidi, Madison, WI) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cell starvation was performed by incubation of Rat-1 fibroblasts for 48 h in glucose-free DMEM, supplemented with 10% FBS and 1 mM sodium pyruvate. For refeeding, cell starvation medium was removed and DMEM containing 25 mM glucose, 10% FBS and 1 mM sodium pyruvate was added, and cells were incubated for 6 h. Generation of the Rat-1 fibroblast clone stably expressing wild type rabbit muscle glycogen synthase (clone WT-4) was described previously (Skurat et al. 2000). WT-4 cells were grown in DMEM, supplemented with 10% FBS and 0.25 mg/mL G418 (10131035, Thermo Fisher Scientific). All media additionally contained 100 U/mL penicillin and 0.1 mg/mL streptomycin.

Immunofluorescence analysis of glycogen in Rat-1 cells

Cells were fixed and permeabilized with methanol for 6 min at −20°C and then slides were incubated with blocking solution (5% BSA and 0.1% Triton X-100 in PBS) overnight at 4°C. In experiments with GYSC, the polypeptide was diluted in blocking solution to a final concentration of 0.28 µg/mL, added to fixed cells and incubated for 2 h at 22°C. After washing three times with PBS buffer containing 0.1% Triton X-100, the slides were exposed to a primary antibody against GST (dilution 1:200) and against other proteins of interest or glycogen in blocking solution for 2 h at room temperature. Antibodies against glycogen, glycogen synthase and c-Myc were used in dilutions 1:100, 1:200 and 1:500, respectively. The bound antibodies were visualized using secondary antibodies conjugated with Alexa Fluor or Texas Red (Invitrogen, Eugene, OR) diluted 1:1000. Nuclei were stained with 1 µg/mL Hoechst 33258 dye. Cells were mounted with ProLong Gold (P36930, Invitrogen) and immunostaining was visualized under fluorescence microscopy. All epifluorescence imaging was acquired on Zeiss Axio Observer Z1 microscope with a c-apochromat_40 water immersion objective (Zeiss, Jena, Germany). Images were processed with Zeiss Axiovision 4.7.

Amyloglucosidase and diastase treatment

After fixation and permeabilization with methanol, cells were incubated with 0.4 mg/mL amyloglucosidase (A7420, Sigma-Aldrich) or

0.5% diastase of malt (D22-100, Fisher Scientific, Fair Lawn, NJ) dissolved in PBS containing 5% BSA for 18 h at room temperature. Cells were then processed for incubation with GYSC and appropriate antibodies.

Glycogen determination

Glycogen content in the Rat-1 fibroblasts was measured by modifications of Suzuki et al. (2001). Rat-1 fibroblasts were seeded in 60-mm plates and grown till 100% confluence. After glucose starvation and refeeding, cell extracts were prepared as described previously (Skurat et al. 1993). Briefly, cells were rinsed twice with 4 mL homogenization buffer (50 mM Tris–HCl pH 7.8, 10 mM EDTA, 2 mM EGTA, 100 mM NaF). Cells were then frozen in liquid nitrogen in 0.3 mL homogenization buffer. After thawing, lysed cells were collected by scraping with a rubber policeman and homogenized with a tissue tearer. Homogenates were centrifuged at 6000 × *g* for 10 min. Glycogen standard solutions and samples of supernatant were hydrolyzed in 30% (w/v) KOH solution in a boiling water bath. After precipitation with ethanol, glycogen was hydrolyzed by amylo-1,6-glycosidase at pH 4.8, and the resulting glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase reaction (Suzuki et al. 2001). Glycogen content was expressed as micrograms of glycogen per milligram of protein. Protein concentration was determined by the Bradford method using BSA as standard (Bradford 1976).

Isolation of fixed single muscle fibers from mouse models of glycogenoses and immunofluorescence microscopy

Two mouse models of glycogenoses were analyzed. The first, $GAA^{-/-}$ mice, which lack lysosomal α-glucosidase (acid maltase) activity, were kindly supplied by Dr. Nina Raben. These mice overaccumulate glycogen in lysosomes and other vesicular stores and have been studied as a model for Pompe disease (Raben et al. 1998, 2003). The second, $Epm2b^{-/-}$ mice were generated in our laboratory (DePaoli-Roach et al. 2010) and lack the malin protein (Chan et al. 2003). Mutations of either *EPM2B* or a second gene, *EPM2A*, which encodes the glycogen phosphatase called laforin (Minassian et al. 1998), cause Lafora disease in humans (Ganesh et al. 2006; Ramachandran et al. 2009). Lafora disease, which is a teenage onset progressive myoclonus epilepsy, appears to result from the overaccumulation of abnormally insoluble deposits of glycogen (Collins et al. 1968; Carpenter and Karpati 1981; DiMauro and Lamperti 2001). Muscle fibers were isolated from mice as described previously (Raben et al. 2009). Briefly, psoas and anterior tibialis muscle samples were removed immediately after sacrifice from $Epm2b^{-/-}$ mice (351 days old) and $GAA^{-/-}$ mice (324 days old), respectively. Muscles were pinned to a Sylgard-coated dish and submerged in 4% paraformaldehyde in PBS for 30 min for fixation at room temperature. Samples were then transferred to cold methanol, and placed at −20°C for 6 min. After washing with PBS, samples were placed in 50% glycerol in PBS and stored at −20°C. Single fibers obtained by manual teasing were incubated for 1 h in a blocking solution consisting of PBS, 0.08% Saponin and blocking reagent from Vector M. O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA). After blocking, fibers were placed in diluent solution containing PBS, protein concentrate (M.O.M. kit) and 0.08% Saponin followed by addition of GYSC to a final concentration 1.1 μg/mL. Fibers were incubated for 16 h at 4°C, washed in PBS containing 0.08% Saponin and incubated with primary antibodies against GST and Lamp1 or glycogen for 2 h at room temperature. Fibers were washed again, incubated with secondary antibody for 1 h at room temperature and mounted in Vectashield (Vector Laboratories, Burlingame, CA) on a glass slide for analysis by immunofluorescent microscopy. For each immunostaining, at least 20 fibers were isolated from psoas or anterior tibialis muscle. Images of muscle fibers from $GAA^{-/-}$ mice were acquired with a confocal/2-photon Leica TCS SP8 system (Leica Microsystems, Inc., Buffalo Grove, IL) by using Leica HC PL APO 40×/1.3 oil immersion objective. Series of sections through the depth of tissue (Z-stacks) were collected by using optimal step size settings (0.35 μm); images were composed of 512 × 512 pixels (92.3 × 92.3 μm^2).

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Conflict of interest statement

None declared

Abbreviations

BB, blocking buffer; BSA, bovine serum albumin; CBM, carbohydrate-binding module; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GSDs, glycogen storage diseases; GST, glutathione S-transferase; GYSC, a fusion protein composed of glutathione S-transferase, a cMyc eptitope and the Stbd1CBM; HRP, horseradish peroxidase; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Stbd1, starch-binding domain containing protein 1.

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