Single fiber analyses of glycogen-related proteins reveal their differential association with glycogen in rat skeletal muscle

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Murphy RM, Xu H, Latchman H, Larkins NT, Gooley PR, Stapleton DI. Single fiber analyses of glycogen-related proteins reveal their differential association with glycogen in rat skeletal muscle. Am J Physiol Cell Physiol 2012; 303(3): C1146–C1155, 2012. First published September 26, 2012; doi:10.1152/ajpcell.00252.2012.—To understand how glycogen affects skeletal muscle physiology, we examined enzymes essential for muscle glycogen synthesis and degradation using single fibers from quiescent and stimulated rat skeletal muscle. Presenting a shift in paradigm, we show these proteins are differentially associated with glycogen granules. Protein diffusibility and/or abundance of glycogenin, glycogen branching enzyme (GBE), debranching enzyme (GDE), phosphorylase (GP), and synthase (GS) were examined in fibers isolated from rat fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscle. GDE and GP proteins were more abundant (~10- to 100-fold) in fibers from EDL compared with SOL muscle. GS and glycogenin proteins were similar between muscles while GBE had an approximately fourfold greater abundance in SOL muscle. Mechanically skinned fibers exposed to physiological buffer for 10 min showed ~70% total pools of GBE and GP were diffusible (nonbound), whereas GDE and GS were considerably less diffusible. Intense in vitro stimulation, sufficient to elicit a ~50% decrease in intracellular glycogen, increased diffusibility of GDE, GP, and GS (~15–60%) and decreased GBE diffusibility (~20%). Amylase treatment, which breaks α-1,4 linkages of glycogen, indicated differential diffusibilities and hence glycogen associations of GDE and GS. Membrane solubilization (1% Triton-X-100) allowed a small additional amount of GDE and GS to diffuse from fibers, suggesting the majority of nonglycogen-associated GDE/GS is associated with myofibrillar/network tissue of muscle rather than membranes. Given differences in enzymes required for glycogen metabolism, the current findings suggest glycogen particles have fiber-type-dependent structures. The greater catabolic potential of glycogen breakdown in fast-twitch fibers may account for different contraction induced rates of glycogen utilization.

glycogen storage disease; glycogen enzymes; single fibers

ONE OF THE MAIN FUEL SOURCES in skeletal muscle for both short-term and prolonged, repetitive contractions is glycogen, a branched polymer of glucose comprising of α-1,4-glycosidic bonds with α-1,6-glycosidic linkages at branch points (6). Glycogen synthesis is initiated by the autoglucosylation of glycogenin and elongated by the activities of glycogen synthase (GS, α-1,4-glycosidic links) and glycogen branching enzyme (GBE, α-1,6-glycosidic shorter branches). GBE excises a segment of existing oligosaccharide on glycogen by cleaving an α-1,4-glycosidic linkage and reforms an α-1,6-glycosidic linkage (2, 7). Muscle glycogen is important for providing glucose as a source of ATP for energy-requiring events like muscle contraction. Glucose is mobilized from glycogen by the concerted action of glycogen phosphorylase (GP) and glycogen debranching enzyme (GDE) acting in reverse to GS and GBE (6). Glycogen granules consist of several tiers of glucose moieties, with 30–45% of the glucose being present in the outer tiers and thereby branched with α-1,4-glycosidic links and requiring GP for utilization. To understand how glycogen as a dynamic fuel source affects skeletal muscle physiology, an improved understanding of the relationship between glycogen-related proteins and the glycogen granule is required. Proteins involved in glycogen metabolism, at least in hepatic tissue, have been documented to interact with the glycogen particle to form a vital dynamic carbohydrate/protein complex (34, 35). However, it is not known to what extent glycogen-related enzymes are associated with glycogen granules in skeletal muscle. Certainly, more than 40 years ago it was noted that it was difficult to extract glycogen from muscle without any glycogen-related proteins “contaminating” the prepared glycogen (14) and more recently we (30) and others (28) have demonstrated the existence of a number of glycogen-associated proteins with glycogen.

Skeletal muscle is heterogeneous in nature being comprised of different fiber types. A typical sample obtained from the vastus lateralis (thigh) muscle from humans consists of a mixed fiber population, broadly termed type I and type II fibers, or slow-twitch and fast-twitch fibers, respectively. The metabolic profile of these fiber types is different, although there is seemingly less difference in humans compared with rodents. Overall, slow-twitch fibers are oxidative while fast-twitch fibers are either oxidative (referred to as type IIa) or glycolytic (type IIx in humans and type IIb in rodents) fibers. Conventionally, the expression of different myosin heavy chain (MHC) isoforms distinguishes fibers as either slow or fast twitch. The cross bridges of a fast MHC isoform consume ATP more rapidly than a slow isoform. In addition, one other major ATP-consuming protein in muscle, the sarcoplasmic reticulum (SR) Ca²⁺-ATPase pump (SERCA), also exists in two isoforms, SERCA1 in fast-twitch fibers and SERCA2 in slow-twitch fibers, and the density of pumps is much higher in fast than in slow-twitch fibers (16). Due to the expression of fast isoforms of MHC and SERCA, fast-twitch fibers are able to both produce force quickly and, equally as important, relax quickly following contraction. In contrast, slow-twitch fibers both contract and relax more slowly. Given the different metabolic profiles of fast-twitch and slow-twitch muscle fibers, they consequently have differing requirements for fuel supply.

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The rate of glycogen utilization is in the order of 0.6–3.6 mmol glycose units·kg\(^{-1}\)·min\(^{-1}\) at 50 and 100% \(\text{Vo}_{2\text{max}}\), respectively, and can increase to 30–50 mmol glycose units·kg\(^{-1}\)·min\(^{-1}\) during maximal dynamic or static contractions (3). Further, there is a marked difference in the glycogenolytic rate between fiber types following intense stimulation, with ~10-fold higher rate in type II vs. type I fibers from human skeletal muscle (10).

Properties of glycogen-related proteins, such as the differences in their association with glycogen, their fiber type distribution, and localization in both oxidative and glycolytic muscle as well as after glycogen usage need to be elucidated. Consequently, in this study we examined the fiber type dependence of many of the known glycogen-related proteins in resting rat skeletal muscle. We hypothesized that the enzymes involved in glycogen breakdown (i.e., catabolic enzymes) would be more highly expressed in fast-twitch compared with slow-twitch skeletal muscle so energy could be rapidly provided during contraction, although those involved in glycogen synthesis (i.e., anabolic enzymes) would not differ between the fiber types. Furthermore, we hypothesized that almost the majority of the glycogen-related proteins would remain associated with glycogen and/or membranous compartments in muscle and that their diffusibility would be augmented following glycogen utilizing in vitro stimulation. To understand how tightly associated the various catabolic and anabolic enzymes are associated with glycogen, we used our technique of allowing diffusible proteins to move out of mechanically skinned quiescent muscle fibers in physiological buffer with or without either amylase, which degrades the glycogen granule at \(\alpha-1,4\) linkages or Triton X-100, which allows proteins associated with membranous compartments to be identified (15, 17). These findings are of particular physiological relevance because the entire pool of a given protein can be assessed. Given there is no loss of any muscle constituent, this approach uniquely allows us to determine the proportion of the various enzymes in the different compartments, relative to the total fiber pool.

**MATERIALS AND METHODS**

**Materials and antibodies.** All chemicals used were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Antibodies were raised against GP and GDE as previously described (25). GBE polyclonal antibody was raised in rabbits against the peptide GBE (189–202) \(\text{C}^{189}\text{CRPKKPRSLRIYES}\) and affinity purified using the same peptide coupled to Sulfolink gel (Pierce, Rockford, IL) as previously described (33). Glycogenin polyclonal antibody was raised in rabbits against the peptide C1147PROPERTIES OF GLYCOGEN-RELATED PROTEINS IN SKELETAL MUSCLEGAGTC-3\(\prime\). The amplified cDNA was cloned into EcoR1 and Xho1 sites of PGEX-6P-3 vector (GE Healthcare), and positive clones were confirmed by sequencing. GST-GBE was expressed in BL21 cells (Merck Chemicals, Darmstadt, Germany) overnight at 16°C. Soluble protein was purified by glutathione Sepharose and Sephadex S-200 gel filtration chromatography (GE Healthcare). The GST tag was removed by prescision protease (GE Healthcare) and the resultant cleavage products repurified by glutathione Sepharose chromatography. Unbound GBE was concentrated to a total volume of 1 ml, and its concentration was determined by protein densitometry against a BSA standard curve.

**Collection of fiber segments from rat skeletal muscle.** With approval of the La Trobe University Animal Ethics Committee, male Long-Evans hooded rats (~6–8 mo old) were killed by overdose with isoflurane (4% vol:vol) in a restricted air space. For the collection of single fibers from quiescent muscle, extensor digitorum longus (EDL) and soleus (SOL) muscles from Long-Evans Hooded rats were rapidly excised and pinned at resting length under paraffin oil and kept cool (~10°C) on an ice pack for fiber skimming. Typically, fibers were collected from 5–10 different animals, unless otherwise specified. In this strain of rat, EDL muscle comprises exclusively glycolytic type IIb and IId fast-twitch fibers (i.e., there are no type IIa or type I fibers) and SOL muscle is exclusively oxidative, consisting of type I and type IIa fibers (26). To examine the fiber type dependency of particular proteins, individual muscle fibers were separated by dissection from a given muscle and fiber segments ~2–3 mm in length and 50–70 \(\mu\)m in diameter (i.e., ~10–15 \(\mu\)g wet weight muscle) were placed directly into SDS loading buffer (0.125 M Tris-\(\text{HCl}\) pH 6.8, 4% SDS, 10% glycerol, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue) diluted 2:1 with physiological solution made of the following (in mM): 129 K\(^+\), 36 Na\(^+\), 1 free Mg\(^2+\) (10.3 total Mg\(^{2+}\)), 90 HEPES, 50 EGTA pH 7.10, and an osmolality of 295 ± 10 mosmol/kgH\(_2\)O. These fiber segments were stored at ~20°C until analyzed.

**Diffusibility of fiber constituents into physiological solution with or without triton or amylase.** By using segments of mechanically skinned single skeletal muscle fibers, it is possible to accurately determine how much of a particular protein is either diffusible in the cytoplasm or bound to some structure within the fiber (15, 19). Fibers were mechanically skinned by removing the surface membrane using fine forceps as shown previously (17). An individual fiber segment from either quiescent or stimulated rat EDL muscle was placed into a small microfuge tube containing 10 \(\mu\)l physiological solution for a specified wash time. A fiber segment is typically 10–15 \(\mu\)g wet weight (~2.5–4 \(\mu\)g total muscle protein). It should be noted that with the skinned fiber preparation any protein that moves away from a binding site is unlikely to find an alternative binding site because of the very large volume that it moved into (i.e., fiber ~10–15 nl vs. total volume 10 \(\mu\)l). Such observations would be seen for purely cytosolic proteins as well as any proteins that are only weakly bound to a cellular structure, and in the present experiments such behavior is that which will be referred to as diffusion. Washes were all at room temperature and wash times were typically 1 and 10 min for proteins; however, for the more diffusible proteins (GBE and GP), a more complete diffusion profile was established using varying wash times comprising additional wash times (e.g., 15, 30, 60, 120, 300 and 600 s). Following washing, the skinned fiber (referred to as “F-s”) was removed from the solution (referred to as “Diff” solution) and collected into 15 \(\mu\)l solution (containing 10 \(\mu\)l physiological solution and 5 \(\mu\)l SDS loading buffer). Five microliters SDS loading buffer were also added to the Diff solution. To further delineate if proteins remaining in the muscle fibers were associated with glycogen, fiber segments following the initial 10 min wash in physiological solution \((n = 9\) from 2 separate animals) were then exposed for 10 min to physiological solution containing amylase (70 ng/\(\mu\)l; Sigma A4268, PMSF-treated amylase solution). Treatment with this amylase would only be active on the \(\alpha-1,4\) linkages; however, we find that such treatment results in all glycogenin (the core protein of glycogen)
becoming measurable, indicating that the treatment separates the glycogen granule sufficiently to release this protein in skeletal muscle (unpublished data, Murphy RM, Xu H). In some instances, and to clarify between passive diffusion or glycogen association, the amylase treatment was for 30 min. Matching skinned fibers (“F-sk-amylase”) and the amylase washes (“Amylase”) were collected as for F-sk and Diff. In some cases, to delineate the diffusion of GP over a 60 s timeframe, skinned fibers were placed directly into the amylase solution and collected after 30 or 60 s. To ascertain if proteins remaining in the muscle fibers were associated with membranes, fibers were washed for 10 min in physiological solution and then treated for a further 10 min in physiological solution containing 1% Triton X-100. Skinned fibers (“F-sk-triton”) and their matched triton wash solutions (“Triton”) were collected as for F-sk and Diff (15, 17). The appearance of SERCA was used as a positive control for solubilization of membranes. Samples were stored at −20°C until analysis by Western blotting.

Preparation of rat skeletal muscle homogenates. For preparation of muscle homogenates, EDL and SOL muscles from Long-Evans Hooded rats (n = 5 each) were homogenized (10:1 wt/vol) in physiological solution except the Na+ concentration was 165 mM and there was no K+ present. For detection of glycogenin, 70 ng/μl amylase (Sigma) was added to muscle homogenates before a 30-min incubation at 37°C. All homogenates were added (2:1 vol/vol) to SDS loading buffer and then further diluted to give a final concentration of ~2.5 μg muscle/μl. Following Western blotting (see below) and to compare between muscle types and between gels, glycogen-related proteins were expressed relative to either actin or MHC and then EDL expressed relative to the average density of SOL samples on a given gel. Glycogen utilizing, fatiguing stimulation of rat EDL muscle. Whole EDL muscles were rapidly excised from animals (n = 4), with one muscle pinned at resting length under paraffin oil and kept cool (~10°C) on an ice pack (control). The contralateral muscle underwent a stimulation protocol so that it would utilize (but not deplete) its glycogen stores. The muscle was attached between a glass hook and an insulated force transducer in an in vitro bath filled with Krebs-ringer solution (containing in mM: 122 mM NaCl, 2.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, with 1.3 mM CaCl2 and 5 mM d-glucose, added on the day of the experiment, pH 7.4) bubbled with 95% O2-5% CO2 at 30°C. Force responses were recorded on a personal computer (Powerlab hardware and Chart 6 software; AD Instruments). The muscle was left to equilibrate for 10 min before being electrically stimulated to contract isometrically. Muscle length and stimulation voltage were varied to give maximum twitch force, and the force frequency characteristics were examined with approximately five tetanic contractions stimulated at various frequencies (5–50 Hz). After this the solution was exchanged with a Krebs-ringer solution without glucose and left for 5 min before the stimulation protocol was started. Initially, the muscle was subjected to a prolonged 50-Hz stimulus until the force plateau. Following a 1-min rest, the muscle received a train of tetani, each elicited with 1 ms pulse at 5 Hz for 500 ms then off for 1,500 ms before repeating (i.e., repeated every 2 s) until peak force dropped to <15% of its initial level (~10 min). Following a 2-min rest the muscle was subjected to a further 0.5-s, 5-Hz stimulus to test whether there was any rapid recovery of the tetanic force, as well as another such stimulus at higher voltage to verify that the stimulating voltage was still supramaximal and finally tested with a prolonged 50-Hz stimulus until the force plateau was reached to assess maximum tetanic force. Immediately afterwards, the muscle was removed from the in vitro bath and pinned at resting length under paraffin oil, as for the control muscle. Mechanically skinned fibers were obtained from both the control and the contralateral stimulated muscles (for which glycogen measurements confirmed that glycogen use had been instigated), and fibers were treated as described for determination of protein diffusibility.

Glycogen assay. Glycogen was measured in representative muscles using amyloglucosidase as previously described (24).

Western blotting. Equal amounts of total protein (as indicated by the abundance of the abundant muscle protein MHC) from whole muscle preparations or fiber and wash sets were separated using either 8 or 9% SDS polyacrylamide gels or 10, 4–15 or 4–20% criterion stain free gels (Bio-Rad, Hercules, CA). Proteins were then transferred from the gels to nitrocellulose. Membranes were exposed to primary antibodies (see Materials and antibodies) overnight at 4°C plus 3–4 h at room temperature, both with rocking. Due to the difference in protein sizes, it was possible to obtain data for GDE (~165 kDa), GP or GS (both 95 kDa), and GBE (~77 kDa) in the same fiber and wash sets by cutting the membranes before primary antibody probes, maximizing the first probe opportunities for each Western blot. Following this, appropriate horseradish peroxidase secondary antibodies were added (1 in 20,000), diluted in 5% skim milk powder in TBS with Tween for ~60 min. Following washes with TBS with Tween, protein bands were visualized using West Femto chemiluminescent substrate (ThermoScientific) and densitometry was performed using Quantity One software (Bio-Rad). The relative positions of molecular mass markers were visualized, and an image was captured under white light before chemiluminescent imaging that was taken without moving the membrane; the separate images were superimposed (such as shown in Fig. 1). The amount of diffusible protein in a given fiber was determined when matched Diff and F-sk samples were run side by side on a gel. The Diff sample contained all the components that had been freely diffusible (i.e., cytosolic) in the skinned fiber segment, and the F-sk sample contained the remaining nondiffusible components. Hence, the percentage of diffusible protein in the fiber was derived simply from the density of the specific immunoreactive band in the Diff lane expressed relative to the sum of the specific bands in the Diff and matching F-sk lanes. When fibers were treated with amylase or triton, the proportion was calculated in a similar manner, however, the sum of the specific bands in the Diff, the wash (Triton or Amylase) and the matching F-sk lanes (i.e., F-sk-triton or F-sk-amylase) were used to ascertain the total pool of a given protein.

Statistics. All results are presented as mean values ± SE unless otherwise stated. Data were analyzed using unpaired or paired Student’s 2-tailed t-tests as indicated, and for fiber type dependence of GP, a Mann Whitney ranked test was performed. To compare treatments, one-way ANOVA with Newman-Keuls post-hoc analyses was performed. Analyses were undertaken using GraphPad Prism version 5.0. Significance was set at P < 0.05.
constituents of single muscle fiber segments were analyzed (i.e., Diff and F-sk, see MATERIALS AND METHODS). In fast-twitch EDL muscle fibers, we found that following a 1-min exposure to the physiological solution the majority of the total GDE, GP, and GS pools remained with the fiber (Fig. 3, A–F). Following a 10-min wash time, there was little further washout of GS and GDE; however, diffusible GP increased to 80% of the total pool (Fig. 3, A–F). GBE diffusibility was determined in fibers collected from SOL muscle, and it was seen that 70% appeared as cytoplasmic constituents within 30 s (Fig. 3, G–H). This amount did not increase when the wash time increased to 10 min (Fig. 3H). Despite the fiber type differences in the abundance of GBE, a similar proportion of the total GBE pool in EDL muscle fibers (compared with SOL muscle fibers) was found to be diffusible, with ~60 and 80% appearing as cytoplasmic constituents within 1 and 10 min, respectively (n = 6 and 10 fibers, respectively, not shown).

Membrane association. Given that GDE and GS were essentially entrapped within the fibers, we conducted experiments to identify whether GDE or GS was associated with membranes in rat skeletal muscle. Treatment of EDL fibers with 1% Triton X-100 resulted in 80% of the integral sarcoplasmic reticulum membrane protein, SERCA1, appearing in the Triton wash confirming the effectiveness of this approach (Fig. 4A and also see Ref. 15). Following the initial washout of the diffusible pools of GDE and GS (30 and 20%, respectively, Fig. 4, F–G), a further ~20% GDE and ~30% GS diffused out of the muscle in the presence of Triton (Triton), with ~60% remaining associated with the fibers for both enzymes (F-sk-triton; Fig. 4).

Glycogen association. To determine whether GDE and/or GS enzymes were associated with glycogen, two approaches were used, namely treatment with amylase or in vitro stimulation (described in Fatiguing stimulation of rat EDL muscle).
Amylase treatment of enriched skeletal muscle glycogen fully unmasks the core glycogen protein glycogenin (Fig. 4E). Following the initial washout of diffusible GDE and GS (Diff), a further 40% (GDE) and 25% (GS) became diffusible in the presence of amylase (Amylase, Fig. 4, C–D, and F–G). Following amylase treatment, the amount of GDE and GS remaining in the fibers was ~15% and ~50% of the total pools, respectively (F-sk-amylase; Fig. 4, F–G). There was no difference in the amount of GDE or GS in the amylase washes when fibers were treated for 10 or 30 min (P > 0.95; n = 9 and 4, for 10 min and 30 min, respectively). We examined GBE diffusion in skinned SOL fibers following 15 or 30 s in physiological solution with or without addition of amylase. There was no apparent difference seen in the amounts of GBE.

Fig. 2. GDE and GP are both more abundant in rat fast-twitch EDL compared with slow-twitch SOL muscle. A: individual fibers dissected from EDL and SOL muscle were separated on 10% criterion stain free gel. The band indicated (#) in stain free gel is migrating at ~95 kDa and being only evident in the EDL lane is likely GP. Membrane was probed for GDE (B) and GP (C). Pooled data show the amount of GDE (D) and GP (E) in EDL fibers expressed relative to the average density of SOL fibers on a given gel. *P < 0.005, two-tailed t-test; n = number fibers analyzed.

Fig. 3. Glycogen-associated enzymes are differentially diffusible in rat EDL muscle fibers. Individual fibers from rat control EDL muscles were mechanically skinned and the diffusible proteins allowed to wash out of the fiber into a physiological buffer (PB) for indicated times (see MATERIALS AND METHODS). Wash solution (Diff) and skinned fiber (F-sk) were collected separately in solubilizing buffer for Western blotting (see MATERIALS AND METHODS). A–D: 8% SDS-PAGE gel was loaded with matched Diff and F-sk. Relative protein in each F-sk lane can be seen from MHC in posttransferred Coomassie-stained gel, which also demonstrated no myofibrillar contamination in the Diff lanes. Western blots show GDE (B), GP (C), and the contractile (and nondiffusible) protein actin (D) in fibers and matched wash solutions collected from control muscle. E: 4–15% stain free gel and Western blot showing GS washout. F: pooled data showing the amount of diffusible GDE (●), GP (○), and GS (●) in EDL fibers collected from resting muscle. Density of protein in Diff expressed as a percentage of the total of a given enzyme in Diff and F-sk (number of fibers, n, for each time point indicated). G: Western blot showing GBE washout in fibers collected from SOL muscle. H: pooled data showing the amount of diffusible GBE in SOL fibers in physiological solution (●) or amylase solution (▼) at indicated time points (see MATERIALS AND METHODS).
between these washes (open triangles in Fig. 3H). Additionally, fibers were initially washed in physiological solution (10 min), where ~80% of the total GBE appeared in Diff and then treated for a further 10 min with amylase solution, leading to ~16% additional diffusion of GBE out of the fiber (not shown, n = 5 fibers), leaving a very little total GBE (~4%) associated with the muscle fibers. Almost the entire pool of GP diffused out of muscle fibers within 10 min; however, this was slower than free diffusion because only ~25% had diffused out of the fibers after 1 min (Fig. 3F). We therefore examined if this was likely due to a weak association with glycogen. Following 30- or 60-s washes in physiological solution with or without amylase (nb. without the initial 10-min period of washout), only a small amount (less than ~10%) more of GP appeared in the 30- and 60-s washes when comparing the fibers exposed to the amylase or physiological solutions (n = 2 fibers per time point, not shown).

**Fatiguing stimulation of rat EDL muscle.** Our fatiguing stimulation protocol resulted in 8.6 ± 1.0 and 18.2 ± 3.7% decrease in force production at 5 and 50 Hz, respectively (n = 4, representative trace shown in Fig. 5A). The latter confirmed the absence of high frequency fatigue, and the deficit observed at 5 Hz indicated that the muscle underwent metabolic fatigue. The protocol was associated with ~50% decrease in muscle glycogen (Fig. 5B), which would be sufficient to remove most outer α-1,4 linkages and expose the first of the α-1,6 branch points but not deplete stores (31). Given the small size of the muscle fiber segments used, it was not possible to measure glycogen (diffusible or total) in the segments (i.e., ~10–15 µg wet weight muscle which would equate to ~1 nmol/µg wet weight at basal levels).

**Protein translocation following stimulation.** Individual fibers were collected from EDL muscles following fatiguing stimulation (n = 4). Subsequent to the stimulating protocol, GP
was rapidly diffusible following fatiguing stimulation, with ~90% of the total GP appearing in the cytoplasmic pool following a 1-min wash (Fig. 5C and compare Fig. 5G and Fig. 3F). This amount was similar to that appearing in the wash after 10 min. Washes of both 1 and 10 min revealed ~50% more diffusible GDE compared with the amount seen in contralateral muscles from the same animals (Fig. 5D and compare Fig. 5G and Fig. 3F). Similar to GDE, GS was more freely diffusible (Fig. 5E and compare Fig. 5G and Figs. 3, E and F), although GBE was less diffusible (Fig. 5F and compare Figs. 5G and 3H) in fibers collected from stimulated compared with contralateral control muscles.

**DISCUSSION**

In this study, we present novel findings demonstrating that glycogen-related proteins are differentially associated with the glycogen granule at both rest and following glycogen-utilizing stimulation. Significantly, the experiments enable the entire pool of a given muscle to be examined and as such the approach uniquely allows the proportion of the various enzymes in the different compartments to be determined relative to the total fiber pool. Such findings present a shift in paradigm (29) and provide physiologically important insights of how glycogen-related proteins are compartmentalized.

Glycogen synthesis is initiated by the autoglucosylation of glycogenin, followed by the concerted action of two enzymes, GS and GBE. To determine the fiber type expression of these enzymes, we measured their immunoreactivity using specific antibodies in whole rat skeletal muscle. Glycogenin has been first “unmasked” by amylase digestion, and GS protein expressions are unchanged between fiber types, suggesting a similar number of glycogen granules as well as similar rate of glycogen chain elongation between fast- and slow-twitch muscles. It has previously been reported that, despite a higher glycogenin activity in rat slow-twitch compared with fast-twitch muscle, there was no difference in the abundance of glycogenin protein between those muscles (9). In contrast, GBE protein expression is significantly higher in slow-twitch compared with fast-twitch muscle fibers. These findings suggest that compared with fast-twitch muscle fibers, slow-twitch fibers have additional branches (~1.6) and are likely more densely packed to produce a more tightly formed granule. This idea is in agreement with data reported previously on a limited number of human fibers, suggesting an ~20% smaller mean particle volume for type I fibers than that for type IIA fibers (12). In contrast, a previous study found no difference in the degree of branching of glycogen from slow- or fast-twitch rabbit muscle by comparing iodine incorporation at two different wavelengths (4), al
though we suspect this method may not be sensitive enough to detect 20–30% changes in glycogen branching.

Both glycogen breakdown enzymes, GP and GDE, especially the former, are weakly expressed in rat slow-twitch fibers in contrast with their higher abundance in fast-twitch muscle fibers (Fig. 2). We predict that an implication of a glycogen granule with more α-1,6 branches would be to slow glycogen breakdown, and in slow-twitch muscle this would be further exacerbated by this low abundance of the catabolic enzymes GP and GDE. Additionally, this suggests that glycogen breakdown occurs at a slower rate in slow-twitch compared with fast-twitch muscle. This agrees with a previous study where single human vastus lateralis muscle fibers following an intense stimulation displayed a ~10-fold higher glycogenolytic rate in type II vs. type I fibers (10). Even so, it is possible for glycogen degradation to occur to a large extent in all fiber types, because following high intensity exercise low glycogen concentrations are obtained in mixed muscle samples postexercise, meaning that considerable glycogen degradation must have occurred in all fiber types (1, 31). We report a notable difference between GP and GDE, with GP ~100-fold more highly expressed in rat fast-twitch fibers compared with slow-twitch fibers, yet GDE was only ~10-fold greater. These data agree well with the glycogen model of an approximate 10:1 ratio of α-1,4 and α-1,6 linkages (13). Together, these results indicate that neither enzyme would be rate limiting during glycogen degradation. Finally, the higher relative abundance of GDE compared with GP we find in slow-twitch muscle (Fig. 2) may facilitate the cleavage of the increased percentage of α-1,6 branches associated with increased GBE expression in these fibers.

Distinct subcellular localizations of glycogen granules have been well established (22), with the major proportion of glycogen found in the intermyofibrillar space (~84%) and smaller proportions in the intramyofibrillar space (~8%) and subsarcolemmal space (~7%). Thus compartmentalization is now thought to be a key feature in regulating glycogen metabolism and particle localization in skeletal muscle (21–23, 27). To further this, we determined whether GBE, GS, GP, and GDE were located within the cytoplasm or bound somewhere within the fiber (e.g., such as at a membrane, on glycogen or on contractile proteins) (15, 17, 19). These experiments were performed in quiescent skeletal muscle as well as muscle that had been intensely stimulated in vitro to utilize intracellular glycogen stores. GBE is essential in glycogen metabolism and one would assume closely associated with glycogen; however, despite this GBE is a diffusible protein (Fig. 3) with ~70% of the total pool diffusing out of the fiber in 30 s, consistent with free diffusion given the molecular mass of GBE (~77 kDa; Ref. 20). GBE diffusion is similar in slow and fast-twitch fibers, regardless of a fourfold greater abundance of GBE in slow-twitch fibers (Figs. 1 and 3). In agreement, GBE was found to not sediment with a protein-glycogen fraction obtained from rabbit skeletal muscle (2) and to not associate with liver glycogen (34), suggesting that the GBE/glycogen association is regulated in some way that to date is unknown. Unexpectedly, there was an apparent decrease of ~20% of the total GBE localized in the cytoplasm following the glycogen utilizing stimulation protocol (Fig. 5), suggesting that the stimulation somewhat alters the localization of GBE. Further studies are necessary to determine if this translocation was to the glycogen granule or to some other structure in the muscle fibers.

In contrast to GBE, ~80% of the total GS pool remains associated within quiescent muscle fibers (Fig. 3). Our first thought was that the majority of GS was associated with glycogen granules, as suggested by the literature (2, 29); however, following glycogen utilizing in vitro stimulation or incubation of the fibers with α-amylase (Fig. 4G) more than half of the total GS pool remains bound within the muscle fibers. These findings indicate that the stimulus of contraction or glycogen breakdown causes only a small shift in the localization of GS (compare Figs. 3F and 5G). The fact that GS was not released with amylase digestion shows unequivocally that GS is not solely associated with glycogen. Overall, these data suggest that only about one-third of the GS is tightly associated with the glycogen particle and that the described dynamic behavior of GS (28) likely constitutes the behavior of half the total GS pool at a given time. We also demonstrate that over half the total GS pool is not membrane associated (Fig. 4G). GS phosphorylation is known to influence its cellular location (27) but was not investigated in this study. GS has been previously reported to accumulate or associate with spherical clusters with a diameter of 200–500 nm (27), which could explain the 50% nondiffusible GS measured in this study.

GP is the rate-controlling enzyme for glycogen degradation that cleaves α-1,4-glucosyl bonds to within four glucose units of each branch point to produce glucose-1-phosphate. This enzyme is regulated by covalent modification and allosteric regulation (6). GP does not contain a known specific glycogen-binding domain, but its structure is curved allowing the catalytic site to orientate towards the surface of the glycogen particle and the regulatory region to orientate towards the cytosol (5). In quiescent muscle, GP is only weakly associated with the muscle fiber (Fig. 3) and behaves like a cytosolic protein in fibers collected from stimulated muscle (Fig. 5). The difference is likely not through a direct association with glycogen since amylase treatment of quiescent muscle fibers did not increase GP diffusibility. While the lack of a glycogen-binding domain explains the rapid dissociation of GP from glycogen bound inside a muscle fiber (Fig. 5), it demonstrates that the GP/glycogen association is a transient process, likely allowing GP to move between glycogen granules present in muscle fibers rather than being tightly associated with a specific granule.

In contrast to GP, only 25% of the GDE pool diffuses from fast-twitch fibers over a 10-min period (Fig. 3). This amount is similar to the 36% diffusible glycogen reported by Goodman et al. (8), and therefore, it is tempting to speculate this diffusible pool of GDE is actually associated with a cytoplasmic pool of glycogen. Following amylase treatment or in vitro stimulation, a further ~50% of GDE diffuses from the fast-twitch fibers (Figs. 4F and 5G), which in combination with the data of Goodman et al. (8) would now suggest that GDE is tightly associated with glycogen. This is consistent with liver glycogen, where GDE has been reported to be part of the hepatic glycogen proteome (34). Consequently, a proportion of the GDE, while most likely being associated with glycogen, must be associated with a structure in muscle cells, preventing it from diffusing out of the fibers. Following removal of the cytoplasmic proteins, exposure of fibers to 1% Triton X-100 resulted in a further 20% GDE diffusing out of the fiber.
Compared with at least 90% of the SR-associated protein SERCA1 washing out of the fiber (Fig. 4, A and F). These data allow us to scrutinize the reported association of glycogen with intracellular membranes and in particular the SR membrane. While GDE by way of its association with glycogen might be in the vicinity of the SR (23), only a small proportion of it could be directly associated with the SR membrane. The method used here, which effectively separates major SR proteins such as SERCA (Fig. 4), ryanodine receptor 1, and calsequestrin (15), provides a spatial resolution that microscopy is less able to provide. While previous studies report a direct association of GDE with isolated the SR (11, 14, 36), using our method of membrane isolation we show this is not the case. We predict the remaining ~20% of nondiffusible GDE may be tightly bound to the intramyofibrillar (21) glycogen fraction as shown previously (30).

In the work presented, the advantage is that the entire pool of muscle constituents were always analyzed side by side; however, this also presents a limitation of the work because the pools obtained are somewhat heterogeneous in nature. The membrane dissociation using Triton would solubilize all membrane pools obtained are somewhat heterogeneous in nature. The membrane dissociation using Triton would solubilize all mem-
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REFERENCES
5. Fletterick RJ, Sprang S, Madsen NB. Analysis of the surface topogra-


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