The Muscle-specific Protein Phosphatase PP1G/R<sub>GL(GM)</sub> Is Essential for Activation of Glycogen Synthase by Exercise*

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In skeletal muscle both insulin and contractile activity are physiological stimuli for glycogen synthesis, which is thought to result in part from the dephosphorylation and activation of glycogen synthase (GS). PP1G/R<sub>GL(GM)</sub> is a glycogen/sarcoplasmic reticulum-associated type 1 phosphatase that was originally postulated to mediate insulin control of glycogen metabolism. However, we recently showed (Suzuki, Y., Lanner, C., Kim, J.-H., Vilardo, P. G., Zhang, H., Jie Yang, J., Cooper, L. D., Steele, M., Kennedy, A., Bock, C., Scrimgeour, A., Lawrence, J. C. Jr., L., and DePaoli-Roach, A. A. (2001) Mol. Cell. Biol. 21, 2683–2694) that insulin activates GS in muscle of R<sub>GL(GM)</sub> knockout (KO) mice similarly to the wild type (WT). To determine whether PP1G is involved in glycogen metabolism during muscle contractions, R<sub>GL(GM)</sub> KO and overexpressors (OE) were subjected to two models of contraction, in vivo treadmill running and in situ electrical stimulation. Both procedures resulted in a 2-fold increase in the GS α+/β glucose-6-P activity ratio in WT mice, but this response was completely absent in the KO mice. The KO mice, which also have a reduced GS activity associated with significantly reduced basal glycogen levels, exhibited impaired maximal exercise capacity, but contraction-induced activation of glucose transport was unaffected. The R<sub>GL(GM)</sub> OE mice are characterized by enhanced GS activity ratio and an ~3–4-fold increase in glycogen content in skeletal muscle. These animals were able to tolerate exercise normally. Stimulation of GS and glucose uptake following muscle contraction was not significantly different as compared with WT littermates. These results indicate that although PP1G/R<sub>GL(GM)</sub> is not necessary for activation of GS by insulin, it is essential for regulation of glycogen metabolism under basal conditions and in response to contractile activity, and may explain the reduced muscle glycogen content in the R<sub>GL(GM)</sub> KO mice, despite the normal insulin activation of GS.

Insulin and contractile activity are major regulators of glycogen metabolism in skeletal muscle. Insulin stimulates glycogen synthesis, and postprandially, ~80% of ingested glucose is taken up by skeletal muscle and converted to glycogen (1, 2). Under these conditions, insulin activates glycogen synthase (GS), as well as glucose transport, via translocation of the GLUT4 transporter (3, 4). Glycogen is a major fuel for the contractile activity of skeletal muscle. During contraction, glycogen is utilized as a source of energy, and it has been demonstrated that, perhaps paradoxically, glycogen resynthesis occurs while glycogen is being broken down (5, 6). Presumably, this represents a mechanism for the rapid replenishment of glycogen stores when exercise ceases (7, 8). Contraction also promotes glucose uptake but most likely via a mechanism distinct from that triggered by insulin (9–12). Insulin-stimulated glucose uptake is blocked by the phosphatidylinositol 3-kinase (PI-3K) inhibitor, wortmannin. In contrast, the increased glucose uptake induced by exercise is wortmannin-insensitive, and the AMP-activated protein kinase (AMPK) (13, 14) has been postulated to play an important role. The period following exercise is characterized by increased glucose uptake and net glycogen synthesis in skeletal muscle, a scenario similar to insulin stimulation of muscle. Despite the fact that the mechanism of GS activation in response to insulin has been extensively studied, the molecular details of both insulin and contraction-induced activation remain mostly unknown.

Glycogen metabolism is controlled largely by the coordinated action of the two enzymes GS and glycogen phosphorylase (Ph). Both enzymes are controlled by covalent phosphorylation and by allosteric effectors (15–17). GS undergoes a complex multisite phosphorylation at nine sites by several protein kinases (17), most notably cAMP-dependent protein kinase, casein kinase I, casein kinase II, GSK-3, and AMPK (18) which generally lead to inactivation. Important regulatory phosphorylation sites are distributed between the NH<sub>2</sub> (sites 2 and 2a) and the COOH termini (sites 3a and 3b) of the GS molecule (19–21). Full activity can be restored to phosphorylated enzyme by the presence of the allosteric activator glucose-6-P (G-6P). Ph is activated by phosphorylation of a single site by phosphorylase

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kinase (22). The less active, dephosphorylated form (Ph) acquires full activity in the presence of the allosteric effector AMP. Dephosphorylation of all three of these key regulatory proteins, GS, Ph, and phosphorylase kinase, is believed to be catalyzed primarily by glycogen-associated phosphatases (PP1Gs) (23).

The three forms of PP1G present in skeletal muscle consist of a catalytic subunit, PP1c, in association with a glycogen-targeting subunit, PTG, R6, or RGL (also called GM (24–28)). RGL is striated muscle specific, whereas the other two subunits are more ubiquitously distributed. PTG may interact with glycogen-metabolizing enzymes (29) and has been implicated in insulin control of glycogen metabolism (25, 30). Adenovirus-mediated overexpression of PTG in cultured human muscle cells results in glycogen accumulation and activation of GS (31). However, the mechanism(s) of regulation of PTG and R6-associated phosphatases are completely unknown.

The muscle-specific phosphatase PP1G/RGL, composed of PP1c associated with RGL (GM), dephosphorylates the regulatory sites on GS as well as on Ph and phosphorylase kinase (23, 27). Phosphatase activity was thought to be regulated hormonally by phosphorylation of site 1 (Ser65) and site 2 (Ser67) on the RGL subunit (32, 33). Phosphorylation of site 1 by the insulin-stimulated protein kinase p90Rsk would enhance association of PP1c to RGL and therefore activity toward GS and phosphorylase kinase (32). Conversely, phosphorylation of site 2 would cause dissociation of PP1c and greatly reduce activity (33).

However, work from several laboratories (34–37) has demonstrated that insulin control of glycogen metabolism does not involve the mitogen-activated protein (MAP) kinase pathway. These studies did not exclude the possibility that insulin could activate PP1G/RGL via other pathways. Our recent observations (38) showed that RGL null mice have significant reductions in basal GS activity and total activity and muscle glycogen content. However, RGL KO and wild type mice exhibited a similar 2-fold activation of GS in skeletal muscle in response to insulin stimulation. These studies clearly demonstrate that PP1G/RGL is not essential for the hormonal control. Instead a novel GS-specific insulin-stimulated type 1 phosphatase was detected (38), indicating that a distinct phosphatase form may be involved. A large body of evidence suggests that insulin activation of GS proceeds via the PI3K/Akt pathway that leads to phosphorylation and inhibition of GSK-3 (39–41). However, GSK-3 alone is not sufficient to account for GS dephosphorylation and activation by insulin (3, 20, 21).

The mTOR, mammalian target for the immunosuppressant drug rapamycin, pathway is also activated by insulin. Rapamycin has been shown to block insulin-mediated activation of GS in muscle and 3T3-L1 adipocytes (35, 39) without affecting insulin-induced inactivation of GSK-3 (42), opening the possibility that mTOR could control GS phosphorylation via a phosphatase. Therefore, insulin may promote glycogen synthesis both via inhibition of GSK-3 and stimulation of a type 1 phosphatase.

Even though PP1G/RGL is not required for insulin-stimulated glycogen synthesis in skeletal muscle, it may be a component of the response to contractile activity. GS has been shown to be regulated differentially in skeletal muscle by insulin and contractions during in vitro and in vivo studies (43–45), with contractions and exercise resulting in a prolonged, substantially greater activation compared with maximal insulin treatment. This suggests that contractions may utilize a separate signaling pathway from insulin to activate GS in response to contractions. Changes in GS activity in human muscle biopsy samples obtained during isometric contractions are associated with changes in protein phosphatase activity (46), but the identity of this enzyme has not been determined. The purpose of the present study was to examine whether the low skeletal muscle glycogen content in RGL KO mice would lead to impaired exercise capacity and to investigate the role of PP1G/RGL in glycogen metabolism during muscle contraction. Our approach was to use two different models of exercise: in vivo treadmill running and in situ muscle contraction in mice that either overexpress or are deficient in the RGL subunit. We demonstrate that GS activation is abolished in RGL KO mice during both in vivo and in situ models of exercise. This finding provides compelling evidence that PP1G/RGL is essential for activation of GS during exercise and may provide a mechanism to explain the reduced muscle glycogen content despite the normal insulin control of GS in the KO mice.

**Experimental Procedures**

**Materials**—Aspergillus niger amyloglucosidase, yeast hexokinase, and yeast glucose 6-phosphate (G-6P) dehydrogenase were purchased from Sigma. Restriction enzymes were from New England Biolabs (Beverly, MA). Taq polymerase was purchased from Promega. Radioactively labeled nucleotides, α-[32P]ATP, [γ-32P]ATP, and [γ-32P]UDP[α-32P]glucose, and [3H]deoxyglucose were purchased from PerkinElmer Life Sciences. Okadac acid was from Roche Molecular Biochemicals. Oligonucleotides were synthesized by Life Technologies, Inc. The transgene vector, p3300MCKCAT, was a gift from Dr. Stephen Hauschka (University of Washington). Monoclonal anti-PP1c antibodies that recognize all isoforms were generously provided by Dr. Jackie Vandenheede (University of Leuven, Belgium) and anti-GS antibodies by Dr. John C. Lawrence, Jr. (University of Virginia). DNA sequencing was done at the Indiana University Biochemistry and Biotechnology Facility (Indianapolis, IN) on an ABI PE-ABI 377XL laser sequencer using fluorescent dyeoxy terminators. Antiserum against rabbit RGL was generated by immunizing rabbits with recombinant His-RGL(-1–453) polypeptide. Reagents for protein assays and electrophoresis were purchased from Bio-Rad. Nitrocellulose membranes for Western blotting were from Millipore (Bedford, MA), and chemiluminescence reagents were from Amersham Pharmacia Biotech. All other chemicals and reagents were obtained from either Sigma or Fisher.

**Generation of RGL OE and KO Mice**—RGL OE mice were generated as described previously (38). For generation of transgenic mice overexpressing RGL in skeletal muscle, the full-length rabbit RGL cDNA (28) was inserted at the Zs/PH site of the p3300MCKCAT transgenic vector that contains the mouse muscle creatine kinase promoter (MCK) (47). The HindIII and Kpn1 DNA fragment that contains the promoter and the cDNA was microinjected into the pronuclei of one-cell inbred C3HeB/FeJ embryos by the established protocols (48). The pups derived from the injected embryos were screened for the presence of the transgene by polymerase chain reaction analysis as described previously (38). Oligonucleotides 5′-GGGAAAGCGTGGAAAGCTCATCTGCT-3′ (ADPR279, sense primer in the mouse MCK promoter) and 5′-CAATGCAAACGTGGAGATAAAACATATTC-3′ (ADPR268, antisense primer in the RGL cDNA) were used. Three positive animals were crossed with DBA/2J strains to establish transgenic lineages. All mice were maintained in temperature- and humidity-controlled conditions with a 12:12 h light/dark cycle and were allowed access to food and water ad libitum. The transgenic mice were generated at the Indiana University School of Medicine Transgenic Facility, directed by Dr. Loren Field.

**Western Blot Analyses**—Animals were sacrificed by cervical dislocation, and skeletal muscles were excised, freeze-clamped in liquid nitrogen, and stored at −80 °C until use. Frozen tissue samples were homogenized in 10 volumes (w/v) of 50 mM Tris/HisCl, pH 7.5 (25 °C), 0.5 mM EDTA, 2 mM EGTA, 100 mM NaF, 1% Triton X-100, 0.1 mM N-p-tosyl-l-lysine chloromethyl ketone, 2 mM benzanidine, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM β-mercaptoethanol, and 10 μg/ml leupeptin and homogenized using a Tissue Tearor model 285-370 (Biocom Products Inc.) at 30,000 rpm for 20 s. The homogenates were then centrifuged at 3,500 × g for 5 min. The resultant supernatants were subjected to SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously (38), utilizing appropriate antibodies. Antibody binding was detected by either 125I-labeled protein A or by enhanced chemiluminescence procedures. Quantification was performed by densitometric scanning of the films. Protein concentration was determined by the Bradford method (49) using bovine serum albumin as standard.
GS and Ph Assays—GS and Ph activity were determined as described previously (38) by measuring incorporation of [14C]glucose from UDP-[14C]glucose into glycogen (50) in the presence or absence of 7.2 mM glucose 6-phosphate and the incorporation of [14C]glucose into glycogen from [14C]glucose 1-phosphate with or without 2 mM 5’-AMP (51), respectively. Activity ratios represent the activity measured in the absence divided by that in the presence of the allosteric effectors, G-6P or AMP.

Glycogen Determinations—Glycogen content in skeletal muscle was measured either by KOH hydrolysis of frozen muscle followed by ethanal precipitation and amyloglucosidase digestion as described (38) or by HCl hydrolysis of muscle homogenates followed by neutralization as described (45). The resulting glucosyl residues were determined spectrophotometrically using a hexokinase/glucose-6-phosphate dehydrogenase-based assay (52).

Treadmill Exercise—Mice were allowed first to become adapted to treadmill (Quinton model 42) running by 5–10 min daily training for 3 days. After removal of food for 5 h, 5–7-month-old animals were then subjected to a “ramp” treadmill running protocol in which the speed of the belt was held constant (0.6 mph), and the incline was increased by 1° every 10 min until the mice were exhausted. Maximal exercise tolerance was determined by the cumulative amount of work (kJ) that the mice performed, calculated as body weight (kg) × vertical distance covered (m) × 9.81. Blood samples were collected from the tail vein for determination of glucose and lactate concentrations before and after treadmill running. After exercise mice were sacrificed by cervical dislocation, and the gastrocnemius and tibialis anterior muscles were removed, snap-frozen in liquid nitrogen, and subsequently processed for determination of glycogen concentrations, glycogen synthase, and phosphorylase activities. An equal number of mice were also sacrificed under basal conditions, and blood and muscle samples were used for comparison to the exercise group. Studies with RGL OE utilized animals from line 3 and WT littermates that had been back-crossed 4 to 5 generations with DBA/2J.

In Situ Muscle Contraction—After a 12-h overnight fast mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (91 mg/kg). The sciatic nerves were bilaterally isolated, and electrodes were placed around each and interfaced with a Grass model S88 electrical stimulation unit (Quincy, MA). Hind limb muscles from one leg were then stimulated to induce tetanic contractions for 15 min (1 train/s, 250 ms, 1 V, 0.2-ms duration), whereas the contralateral limb served as a non-stimulated control. Immediately following contractions, both extensor digitorum longus (EDL) muscles were removed and used for measurement of [3H]2-deoxyglucose uptake, as described previously (53), and glycogen content. Tibialis anterior and gastrocnemius muscles were also removed, snap-frozen in liquid nitrogen, and subsequently processed for determination of GS and Ph activities, glycogen content, and Western blotting analyses.

Statistical Analyses—All data are presented as means ± S.E. of the number of animals indicated in the figure legends. Statistical significance was assessed by analysis of variance or unpaired Student’s t test.

RESULTS

Impaired Exercise Capacity in RGL KO Mice—The RGL knockout mice are characterized by low skeletal muscle glycogen content (38). Since glycogen is a major source of energy during exercise, a critical question was whether these animals would be able to sustain exercise. Treadmill running showed that the knockout mice had a 60% lower work capacity as compared with wild type littermates (Table I). Consistent with the previous report (38), basal muscle glycogen content in RGL knockout was significantly reduced. Since exercise has been shown to decrease glycogen more readily in type II (fast twitch) than in type I (slow twitch) muscle fibers (54), representatives of both muscle types were analyzed. Although exhaustive treadmill exercise depleted muscle glycogen by 30–50% in WT mice, the low basal glycogen content in the KO mice was not further reduced by exercise (Fig. 1). Similar results were obtained with two different muscles, tibialis anterior (Fig. 1A), a white muscle, and gastrocnemius (Fig. 1B), which is of mixed red and white fiber types. No differences between WT and KO mice were observed with respect to blood glucose or lactate concentration during exercise (Table I).

Altered Glycogen Metabolism during Exercise in RGLKO Mice—Other phenotypic characteristics of the RGL knockout mice are reduced GS −→ G-6P and increased Ph −→ AMP activity ratios (38). After treadmill exercise of WT mice, Ph activity changed in a muscle-specific manner. In the tibialis anterior (Fig. 2A), treadmill running increased the −→ AMP activity ratio, whereas in the mixed gastrocnemius (Fig. 2B) the activity ratio was reduced. Exercise had no significant effect on Ph activity in either muscle of the KO mice, possibly because of the close to maximal activation of the enzyme under basal conditions.

GS activity state was also examined. As reported previously (38), the basal activity in WT mice was significantly higher than in KO animals, and treadmill running elicited an increase in GS −→ G-6P activity ratio in both tibialis anterior (Fig. 2C) and gastrocnemius (Fig. 2D) muscles of WT mice. In contrast, maximal treadmill exercise did not increase GS activity in the muscles from KO mice. Consistent with these findings, analysis of GS protein revealed an increased electrophoretic mobility in samples from exercised WT but not in KO mice, indicating that GS dephosphorylation was impaired in the latter animals (Fig. 2, E and F). GS protein was significantly reduced in both mixed gastrocnemius and tibialis anterior muscles of KO mice and exhibited a slower mobility, consistent with higher phosphorylation state and decreased activity ratio. These findings provide compelling evidence that PP1G/RGL is necessary for the dephosphorylation and activation of GS that occur in skeletal muscle during exercise.

Effect of In Situ Muscle Contraction on GS Activity in WT and RGLKO Mice—It was possible that the lack of GS activation and glycogen depletion observed in the KO mice following exercise may have been due to the 60% lower work capacity. Therefore, glycogen metabolism was also examined during electrically stimulated in situ muscle contraction in order to standardize the amount of muscular work performed by the different groups. In situ contractions induced an increased GS activity in both the tibialis anterior (Fig. 3C) and gastrocnemius (Fig. 3D) muscles of WT mice. Although there was a tendency for decreased glycogen levels in the gastrocnemius, the effect was not statistically significant (Fig. 3B). Similar to in vivo treadmill exercise, in situ contractions did not reduce muscle glycogen content (Fig. 3, A and B) or increase glycogen synthase activity in muscles from KO mice (Fig. 3, C and D), nor did it change Ph activity (Fig. 3F). These data are consistent with the results of the in vivo experiments, demonstrating that PP1G/RGL is essential for the activation of GS that occurs in response to muscle contractions. To monitor glucose uptake,
we analyzed a small muscle suitable for this analysis, the extensor digitorum longus (EDL). Following the contraction protocol, \[^3\text{H}\]2-deoxyglucose uptake rates were equally increased over basal levels in both groups (Fig. 3E), indicating that RGL does not control glucose transport either by contraction or by insulin (38). Glycogen was determined in the same extracts (Fig. 3A), yielding similar results to those with gastrocnemius (Fig. 3B).

Characterization of RGL-overexpressing Mice—Muscle-specific overexpression of RGL was driven by the \(-3,300\) to \(+7\) promoter sequence of the mouse creatine kinase gene (47) which directs expression mainly in skeletal muscle and to a low level in heart. Of the three lines carrying the RGL transgene, one did not overexpress the protein, whereas the other two expressed up to 10-fold the level observed in rabbit, with a concomitant 6-fold increase of the endogenous PP1c (Fig. 4) in skeletal muscle. Since antibodies raised against the rabbit RGL, the form expressed in the transgenic mice, do not recognize the mouse protein, the level of expression is expressed as a percentage of the level of normal rabbit skeletal muscle. Western analysis utilizing antibodies to the mouse protein indicated that the level of the endogenous RGL is not changed (data not shown). Solubilization of RGL required the presence of Triton X-100 in the homogenization buffer, confirming that the pro-
tein is associated with membranes (28). The increased endogenous PP1c is consistent with the decreased levels observed in the RGL KO mice (38) and supports the hypothesis that expression of the regulatory subunit stabilizes the catalytic subunit.

Neither Ph activity ratio nor total activity were significantly affected by overexpression of RGL (data not shown). Phosphatase activity measured with 32P-Ph as a substrate showed only 50% increase over basal. GS/G-6P activity ratio increased from 0.28 to 0.51 without changes in the total activity or protein in muscle extracts (Fig. 5, A and C), and the glycogen levels were enhanced by 3–4-fold (Fig. 5B). Consistent with the increased GS activity ratio, an increased electrophoretic mobility of the protein was detected, indicative of dephosphorylation (Fig. 5C). These observations provide further evidence that PP1G/RGL is linked to glycogen metabolism in skeletal muscle.

Preliminary glucose uptake determinations using isolated EDL muscle did not reveal significant effects on glucose transport in transgenic mice as compared with wild type littermates. To determine whether glucose disposal was altered, glucose and insulin tolerance tests were performed. Fasted and fed state blood glucose levels were in the normal range. Similarly, no differences were detected between wild type and overexpressing mice in glucose clearance following glucose load or insulin challenge (data not shown).

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Increased Muscle Glycogen Content in the RGLOE Is Not Associated with Enhanced Exercise Tolerance—Despite the differences in basal muscle glycogen content, RGL OE and WT mice displayed a similar physical work capacity during the treadmill running protocol (Table II). Blood glucose concentrations were reduced below basal levels in both groups following.
FIG. 5. GS activity ratio and expression and glycogen content in WT and RGL OE mice. Glycogen synthase activity ratio (A) and glycogen content (B) were determined in skeletal muscle of WT and RGL OE mice. C, upper part shows a Western blot of representative extract samples (20 µg of protein/lane), and the lower part shows quantitation of the autoradiogram. Note the increased electrophoretic mobility of GS in the RGL3-overexpressing mice. **, *p < 0.01 versus WT, n = 5–12 per group. GS–P, glycogen synthase with greater mobility indicative of a lower phosphorylation state; GS–P, glycogen synthase with lower mobility indicative of a higher phosphorylation state. Other abbreviations are as in the legend to Fig. 4.

The major finding of the current investigation is that the marked increase in GS activity in skeletal muscle in response to both in vivo exercise and in situ electrically induced contraction is abolished in mice lacking the RGL regulatory/glycogen-targeting subunit of PP1G. In exercised WT mice, the changes in GS activity state are accompanied by increased electrophoretic mobility of the protein, diagnostic of dephosphorylation. To date, the mechanism by which GS is activated during muscle contractions has not been elucidated. It is well known that the rate of glucose uptake is greatly increased in contracting skeletal muscle, which is associated with an accumulation of G-6P within the myoplasm (7). Increased G-6P concentration may provide a mechanism for regulating GS during exercise via its allosteric effect on activity and also by rendering GS more susceptible to dephosphorylation by phosphatases (55). However, GS was not activated in RGL KO mice despite the normal rates of basal and post-contraction glucose uptake compared with WT mice, suggesting that RGL mediates some other mechanism by which contraction induces GS activation. Decreased phosphorylation of GS could be due to decreased kinase activity, increased phosphatase activity, or both. Changes in GS activity in skeletal muscle biopsies from exercising humans have been associated with changes in the activity of a GS phosphatase (46). However, these studies were correlative, and the enzyme form was not identified. The results of the in vivo and in situ experiments with RGL OE mice provide the first direct evidence that PP1G/RGL is a phosphatase involved in the signaling cascade leading to activation of glycogen synthase during exercise and additionally demonstrate that it is an essential component of this control.

PP1G/RGL was originally proposed to mediate insulin-stimulated glycogen synthesis in skeletal muscle via the MAP kinase signaling pathway (32). Although subsequent studies using epidermal growth factor (35), MAP kinase/extracellular signal-regulated kinase inhibitors (36), and RSK2 KO mice (37) argued against an involvement of the MAP kinase signaling cascade, they did not exclude the possibility that PP1G/RGL mediated the insulin response. However, recent work (38) demonstrating normal activation of GS by insulin in RGL KO mice clearly showed that PP1G/RGL is not required. Furthermore, although insulin may increase phosphorylation of RGL at Ser48 and Ser67 in cultured cell systems (56), it does not affect phosphorylation at these sites in vivo (57) or mouse (38) skeletal muscle. Activation of GS by PP1G/RGL during muscle contraction appears to be a mechanism that is not shared with the insulin signaling pathway, and insulin stimulation of GS appears to involve a distinct phosphatase specific for GS (38). A large body of evidence suggests that insulin and exercise control glucose uptake by distinct mechanisms, mediated by IP-3/3K/Akt and AMPK, respectively. Therefore, in a similar way, insulin and contraction may stimulate metabolism.
Muscle glycogen concentrations under basal conditions and following treadmill exercise in WT and RGL OE mice. Glycogen content was determined as in the legend to Fig. 1 under basal conditions (open bars) and after exhaustive treadmill running (filled bars) of WT and RGL OE mice. *, *p = 0.003 versus basal; #, *p < 0.001 versus WT. n = 3–4 per group.

**Fig. 6.** Effect of in situ electrical stimulation on glycogen content and GS and glucose uptake in WT and RGL OE mice. Glycogen content (A) and GS activity ratio (B) were measured in unstimulated (open bars) and electrically stimulated (filled bars) contralateral EDL and tibialis anterior muscles, respectively. Glucose uptake was determined in EDL muscle, as described under “Experimental Procedures.” *, *p < 0.01 versus basal; #, *p < 0.001 versus WT. n = 9–10 per group. Abbreviations are as in the legend to Fig. 1.

GS through activation of different protein phosphatases. Whether RGL is a downstream effector of AMPK is an interesting question that is currently under investigation. However, other pathways, such as elevation of intracellular Ca2+ concentration, cannot be excluded. It is unlikely that RGL is controlled by the MAP kinase pathway, since the MAP kinase inhibitor PD98059 did not alter contraction-induced increases in muscle glycogen synthase activity (58).

Although our data indicate that PP1G/RGL is an obligatory component of an exercise-activated signaling pathway that is not shared by insulin, we cannot exclude the involvement of other mechanisms to activate glycogen synthase during muscle contraction. Specific enzymes in such pathways could represent points of convergence between insulin and exercise signaling. For example, insulin activates a PI-3K/Akt-dependent pathway that inhibits GSK-3 (39–41). We have previously shown (45) that both GSK-3α and -β isoforms are inhibited in rat skeletal muscle during treadmill exercise. However, as for insulin signaling, GSK-3 alone cannot account for GS activation, since modulation of activity requires changes in the phosphorylation of NH2-terminal phosphorylation sites that are not substrates for GSK-3 (3, 20, 21). Also, GSK-3 expression is not altered in the RGL KO mice (38). Nevertheless, it is possible that exercise-induced inhibition of GSK-3 plays an ancillary role in activating GS, in a pathway shared with insulin. Further work is required to identify what upstream effectors regulate PP1G/RGL and GSK-3 during muscle contractions.

Both the RGL-overexpressing and KO mice exhibit differences in basal muscle glycogen concentrations compared with their respective wild type littermates. The RGL-overexpressing animals have significantly increased glycogen, whereas glycogen is severely depleted in the KO mice. Analysis of GS and Ph activities in muscle extracts indicates that these differences are due to alterations in the rates of both synthesis and degradation. Overexpression of related phosphatase-targeting proteins, such as PTG, has been shown to promote glycogen synthesis in hepatocytes in culture (30, 59) and in vivo (60) and in cultured human muscle cells (31), presumably by increasing phosphatase activity toward glycogen-bound substrates. Although a role for PTG in insulin control of glycogen metabolism has been proposed, our work indicates that PTG is not involved in regulation by contraction. Most importantly, the present study emphasizes the fact that the various forms of PP1G are not redundant since neither the PTG nor the R6 forms of PPIG complemented the inability of the RGL KO mice to activate GS in response to contraction. Also, the fact that RGL overexpression did not alter exercise performance or the ability to activate GS indicates that gene dosage is not a critical factor. Various reports (62–65) have argued that the intracellular glycogen content is inversely related to insulin- and exercise-induced stimulation of glucose transport and GS. However, in the RGL OE and KO animals, the muscle glycogen content ranged from ~4-fold above normal to ~5-fold lower, without any observed differences in glucose uptake, whether basal, contraction-induced, or insulin-induced (38). Therefore, our results demonstrate that glycogen content by itself is not sufficient to determine the activation potential of the two likely rate-determining steps of its synthesis.

Muscle glycogen is a primary fuel source during muscle contractions and may limit the duration of certain types of activities (66). Thus it was perhaps not surprising that the RGL KO mice had a 60% reduction in work capacity during maximal treadmill exercise. It is interesting that the low basal muscle glycogen concentrations in the knockouts, which were similar...
to post-exercise values in the WT littermates, were not further reduced either by maximal treadmill exercise or in situ contractions. Skeletal muscle appears to contain different pools of glycogen that are subject to different mechanisms of metabolic regulation, and the muscle may exist that is resistant to depletion by contractions (67). In humans, the rate of glycogenolysis during exercise is significantly reduced in muscle with a low pre-exercise glycogen concentration (68). Therefore, it seems likely that the low basal glycogen content in the RGL KO mice causes other fuel sources (e.g., blood glucose, amino acids, and fatty acids) to become the primary fuel source during exercise. The higher blood glucose levels observed in the RGL-overexpressing mice after exercise lend some support to this hypothesis. Additionally, branched chain amino acid oxidation has been shown to be inversely related to post-exercise muscle glycogen content in humans following prolonged cycle ergometry (69). If RGL KO mice had an increased reliance on alternate fuel substrates, such as amino acids and fatty acids, in an attempt to preserve a “threshold” glycogen store during exercise, then a reduced rate of ATP generation compared with anaerobic or aerobic glycolysis may partly explain the reduced maximal exercise tolerance. Although previous measurements of 24-h respiratory rates (VO2/VO2CO2) indicated that resting fuel substrate utilization in RGL KO and WT mice was the same (38), there could be differences during exercise, a possibility that merits further study. It has been reported that depletion of RGL in L6 cells affects cell differentiation (61), and abnormal muscle development could, in principle, contribute to impaired exercise capacity. No comparable phenomenon occurs in whole animals, however, since histological analysis of various muscle types, including soleus, EDL, and gastrocnemius, from RGL KO mice revealed no evidence for morphological defects.

In conclusion, although not required for GS stimulation by insulin (38), RGL is an essential mediator of GS activation in response to skeletal muscle contractions. Therefore, PP1/GS/GSK-3 may be a component of a signaling cascade that is unique to exercise. Work is in progress to determine the mechanism(s) and the components of the signaling pathway that regulate RGL and GSK-3 during exercise.

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FIG. 8. Effect of in situ electrical stimulation of gastrocnemius muscles on GS and Ph activity ratios and on glycogen content in WT and RGL OE mice. GS (A) activity ratio and glycogen content (B) were measured in unstimulated (open bars) and electrically stimulated (filled bars) contralateral gastrocnemius muscles. C shows a Western blot of GS from representative basal and electrically stimulated (ES) WT and RGL OE mice. Note the increased GS electrophoretic mobility following in situ contraction, both in the WT and the OE animals. n = 11 (A) or 8 (B) per group. GSp, glycogen synthase with greater mobility indicative of a lower phosphorylation state; GS, glycogen synthase with lower mobility indicative of a higher phosphorylation state. **, p < 0.001 versus basal; #, p < 0.0001 versus WT. RGL OE line 3.
