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Allosteric Regulation of Glycogen Synthase Controls Glycogen Synthesis in Muscle

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SUMMARY

Glycogen synthase (GS), a key enzyme in glycogen synthesis, is activated by the allosteric stimulator glucose-6-phosphate (G6P) and by dephosphorylation through inactivation of GS kinase-3 with insulin. The relative importance of these two regulatory mechanisms in controlling GS is not established, mainly due to the complex interplay between multiple phosphorylation sites and allosteric effectors. Here we identify a residue that plays an important role in the allosteric activation of GS by G6P. We generated knockin mice in which wild-type muscle GS was replaced by a mutant that could not be activated by G6P but could still be activated normally by dephosphorylation. We demonstrate that knockin mice expressing the G6P-insensitive mutant display an ~80% reduced muscle glycogen synthesis by insulin and markedly reduced glycogen levels. Our study provides genetic evidence that allosteric activation of GS is the primary mechanism by which insulin promotes muscle glycogen accumulation in vivo.

INTRODUCTION

Elevated blood glucose levels after a meal are rapidly returned to normal, and during starvation blood glucose is maintained only slightly below normal. Such a sophisticated control system is essential to prevent serious dysfunctions such as loss of consciousness due to hypoglycaemia and toxicity to peripheral tissues in response to persistent hyperglycaemia (Wasserman, 2009). Glucose is distributed into multiple tissues of the body where it can be oxidized to produce energy or stored as the polysaccharide glycogen. In humans, the major cellular mechanism for disposal of ingested glucose is insulin-stimulated glucose transport into skeletal muscle, with the majority of glucose that enters muscle fibers being converted to glycogen (Shulman et al., 1990). Therefore, impaired insulin-stimulated muscle glucose transport and glycogen synthesis are implicated in the pathogenesis of insulin resistance and type 2 diabetes (Shulman, 2000).

Insulin stimulates glucose transport by promoting the translocation of the glucose transporter, GLUT4, from intracellular compartments to the cell surface. The resulting increase in glucose transport and phosphorylation of glucose by hexokinase leads to an increase in the intracellular concentration of glucose-6-phosphate (G6P). G6P is mainly used for the synthesis of glycogen in resting muscle, while it is largely metabolized in the glycolytic pathway during contraction. During glycogen synthesis, G6P is converted to UDP-glucose and the glucosyl unit from UDP-glucose is used to elongate a nascent glycogen chain through α-1,4-glycosidic bonds by the action of glycogen synthase (GS), which is stimulated by insulin (Roach, 2002). This activation of GS in response to insulin occurs in minutes and was the first example of regulation of a specific enzyme by insulin in cells (Villar-Palasi and Larner, 1960). Despite decades of intensive research in this field, the molecular mechanism(s) whereby insulin activates GS in vivo is controversial, although several models have been proposed (Lawrence and Roach, 1997).

In the early 1960s two mechanisms were identified by which GS activity could be regulated: allosteric activation by G6P (Leloir et al., 1959) and covalent phosphorylation (Friedman and Larner, 1963), which inhibits the enzyme. Insulin activates GS through allosteric regulation by elevating the levels of G6P via glucose transport and also phospho-dependent mechanisms by promoting the conversion of GS from a highly phosphorylated (low-activity) form to a less phosphorylated (high-activity) form through the protein kinase B (PKB, also known as Akt)-dependent inactivation of glycogen synthase kinase 3 (GSK3) (McManus et al., 2005). Dephosphorylation results in significant changes in the kinetic properties of GS, decreasing the Km for the substrate UDP-glucose and the $A_{0.5}$ for G6P (Friedman and Larner, 1963; Jensen and Lai, 2009). Thus, it is thought that insulin stimulates GS through the complex interplay between phosphorylation and allosteric regulation. A major obstacle to gaining molecular insight into how insulin regulates GS in vivo is a lack of understanding of the relative importance of these two regulatory mechanisms, allosteric and phospho dependent, in controlling GS activity.

To understand the contribution that phospho-dependent activation of GS plays in insulin-stimulated muscle glycogen synthesis, we previously generated knockin mice expressing

constitutively active mutants of GSK3 (both α and β isoforms) in which the PKB phosphorylation sites on GSK3α (Ser21) and GSK3β (Ser9) were substituted by Ala (GSK3α/β$^{S21A/S21A/S9A/S9A}$) (McManus et al., 2005). In GSK3α/β$^{S21A/S21A/S9A/S9A}$ animals, we found that insulin failed to inactivate muscle GSK3 and hence promote GS activation by dephosphorylation. Strikingly, we found that insulin-stimulated glycogen synthesis and the levels of glycogen in skeletal muscle were similar between wild-type and GSK3α/β$^{S21A/S21A/S9A/S9A}$ mice (Bouskila et al., 2008). These observations led us to hypothesize that the allosteric activation of GS by G6P plays a major role in glycogen synthesis, which would compensate for the lack of phospho-dependent activation of GS by insulin in the GSK3α/β$^{S21A/S21A/S9A/S9A}$ mice. While this is an attractive hypothesis, no robust cell-based assays or genetic models are available to establish the in vivo role that allosteric activation of GS plays in muscle glycogen synthesis.

To this end, we aimed to identify critical residues for the G6P-mediated allosteric activation of GS, which could be exploited to generate a knockin mouse expressing a G6P-insensitive GS mutant. Here we report genetic evidence that the allosteric activation of GS is the primary mechanism by which insulin promotes glycogen synthesis in muscle.

RESULTS

Identification of Critical Residue(s) Important for Allosteric Activation of GS by G6P

Alignment of the sequences of *Saccharomyces cerevisiae* and mammalian GS isoforms revealed that there is a highly basic segment present at the C terminus of both yeast and mammalian GS (Figure 1A). Pederson et al. performed scanning mutagenesis analysis using the major *Saccharomyces cerevisiae* GS isoform, Gsy2p, and reported that mutation of multiple arginine residues within this conserved basic segment to alanine blocked the allosteric activation by G6P (Pederson et al., 2000). They subsequently demonstrated that corresponding mutations had a similar effect on rabbit muscle GS (GYS1) (Hanashiro and Roach, 2002). However, ectopic expression of the G6P-resistant rabbit muscle GS mutant R578A/R579A/R581A (all three of the indicated Arg residues changed to Ala) in COS-1 cells revealed a reduced expression level and a markedly higher basal activity when assayed in the absence of G6P relative to the wild-type enzyme (Hanashiro and Roach, 2002). Substitution of multiple Arg residues to Ala likely disrupted the three-dimensional structure, resulting in destabilization and dysfunction of the enzyme. In the current study, our initial goal was to identify a G6P-insensitive muscle GS mutant that expressed at normal levels in cells and also displayed unaltered phospho-dependent activity. We generated a series of mutants in which individual Arg residues or a combination of two or three Arg residues in the highly conserved basic segment (Figure 1A) were changed to Ala or Glu. We transfected HEK293 cells, which express only trace amounts of endogenous GS (Figure 1B), with these GS mutants together with glycogenin, a specialized initiator protein in glycogenesis which binds to and enhances expression of soluble GS (Skurat et al., 1993). Thirty-six hours following transfection of these mutants, cell extracts were generated, and expression of GS and glycogenin, as well as GS activity, was assessed (Figure 1B). Consistent with a previous report (Hanashiro and Roach, 2002),

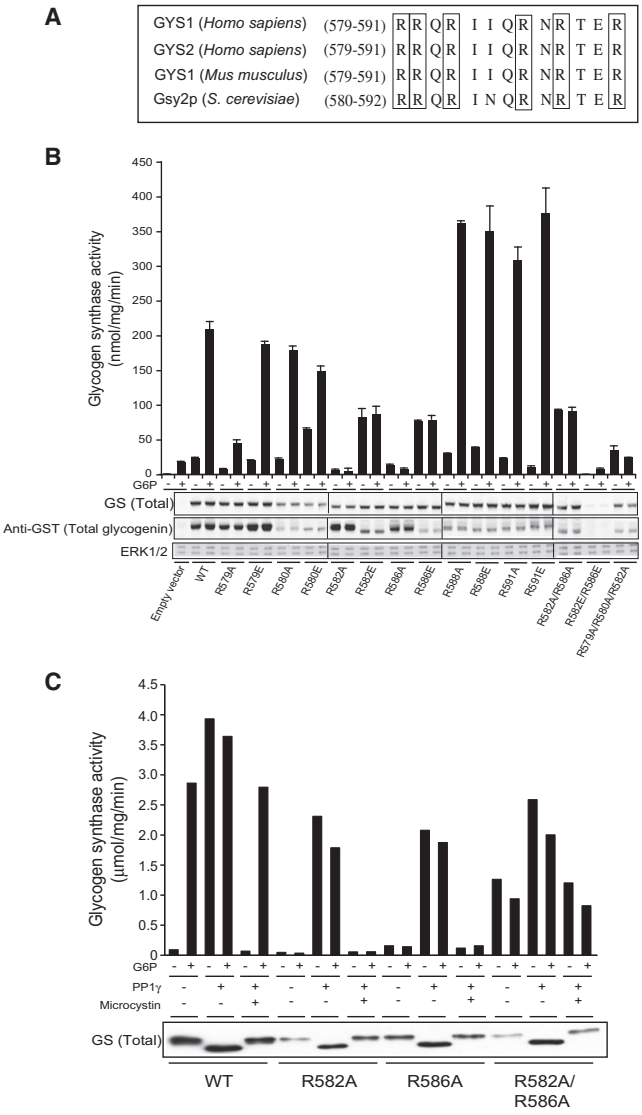

Figure 1. Mutagenesis and Biochemical Analyses to Identify Glucose-6 Phosphate-Insensitive Glycogen Synthase Mutants

(A) Amino acid sequence alignment of putative G6P sensitizing residues of mammalian and yeast GS. Arginine (R) residues located in the G6P-sensitizing region shown in (A) were mutated individually or in combination to either alanine (A) or glutamic acid (E).

(B) Constructs expressing wild-type and the indicated GS mutants were cotransfected with GST-tagged glycogenin. Cell extracts were prepared and equal amounts of protein were immunoblotted with the indicated antibodies or assayed for GS activity in the presence and absence of 10 mM G6P. The ERK1/2 kinases were immunoblotted as a loading control. Results are representative of two independent experiments.

(C) Equal quantities of purified GS mutants were dephosphorylated in vitro using PP1γ. The PP1 inhibitor microcystin-LR was used as a negative control. Mock and PP1γ-treated GS mutants were assayed for GS activity in the presence or absence of G6P and immunoblotted to confirm equal loading and assess the phosphorylation status of GS. Results are representative of two independent experiments.

we observed that the R579A/R580A/R582A triple mutant was insensitive to G6P and had modestly reduced expression compared to the wild-type enzyme. We also observed that

several other mutants including single R582A, R582E, R586A, R586E, R586A, and double R582A/R586A and R582E/R586E mutants were also resistant to G6P (Figure 1B). However, the R582E/R586E mutant displayed markedly reduced cellular expression, and single R582E, R586E, and double R582A/R586A mutants had ~3-fold higher basal activity compared to wild-type when assayed in the absence of G6P. We noticed that the expression of glycogenin was reduced (e.g., R580A, R580E, R586E) and gel mobility increased (e.g., R582E, R586E) when particular GS mutants were cotransfected (Figure 1B). We are unable to provide an accurate molecular explanation for these alterations in glycogenin. We speculate that certain amino acid substitutions may destabilize the interaction between GS and glycogenin, resulting in reduced expression of both enzymes (e.g., R580, R580E, R582E/R586E, R579A/R580A/R582A). Alternatively, amino acid substitution may have caused a change in intrinsic activity/conformational change in the GS/glycogenin complex, resulting in undefined posttranslational modifications (e.g., glycosylation, phosphorylation) of glycogenin by an unidentified mechanism(s). Among the mutants analyzed, only two single-point mutants (R582A and R586A) retained normal levels of expression (soluble) and catalytic activity (in the absence of G6P) relative to the wild-type enzyme (Figure 1B). While a trace amount of wild-type and mutant GS, as well as glycogenin, was found in the particulate fraction of cell lysates, this also occurred at similar levels (data not shown). We then examined if these point mutants (R582A and R586A) possessed normal phospho-dependent activity. Since GS proteins overexpressed in HEK293 cells were highly phosphorylated, we determined if R582A and R586A mutants could be activated to the same degree as wild-type by dephosphorylation in vitro. Purified wild-type, R582A, R586A, and R582A/R586A GS mutants were incubated in the presence of type 1 protein phosphatase (PP1) γ. Wild-type GS was efficiently dephosphorylated by PP1γ, as evidenced by faster gel mobility (Figure 1C), complete loss of immunoreactivity to a phospho-specific GS antibody (data not shown), and the fact that catalytic activity of the dephosphorylated enzyme was no longer dependent on G6P (basal activity equivalent to G6P-saturated activity) (Figure 1C). These effects were blocked by the PP1 inhibitor microcystin-LR (Figure 1C). Notably, PP1γ promoted a robust activation of G6P-resistant R582A and R586A mutants with or without G6P close to the levels observed in wild-type, which was associated with a marked dephosphorylation as judged by gel mobility shift of total GS (Figure 1C) and staining with phospho-specific antibodies (data not shown). Catalytic activity of the double R582A/R586A mutant was also increased by PP1γ-dependent dephosphorylation, although a markedly higher activity in the absence of G6P compared to wild-type was observed (Figures 1C and 1A). Taken together, we have identified key Arg residues that are essential for the allosteric activation of GS by G6P. The single residues, Arg582 and Arg586, can be substituted for alanine to generate mutant enzymes that are completely resistant to G6P yet retain normal cellular expression and phospho-dependent activity when expressed in HEK293 cells.

Generation and Characterization of G6P-Insensitive GS Knockin Mice

To explore the physiological roles that allosteric activation of GS by G6P might play in muscle glycogen metabolism in vivo, a knockin mouse was generated in which the codon for arginine 582 of *GYS1* (muscle isoform) was modified to encode alanine (Figures 2A–2C). When the litters from $GS^{+/R582A}$ breeding pairs were genotyped, we observed that $GS^{R582A/R582A}$ mice were born at a slightly lower rate (18.4%) than the expected normal Mendelian frequency (25%). Regardless, $GS^{R582A/R582A}$ knockin mice displayed no overt phenotype, and growth curves from 5 to 18 weeks of age in both males and females indicated that these animals were of normal size and weight (see Figure S1A available online). Likewise, $GS^{R582A/R582A}$ knockin mice consumed a similar amount of food compared to the wild-type (Figure S1B).

We determined if expression of mutant R582A GS in $GS^{R582A/R582A}$ animals was comparable to wild-type GS in several tissues. Immunoblot analysis revealed that there was no difference in the levels of muscle GS between wild-type and $GS^{R582A/R582A}$ knockin mice in various skeletal muscles and other tissues (Figure 2D). We also observed that >98% of GS protein in muscle lysates from wild-type and $GS^{R582A/R582A}$ knockin mice was recovered in the soluble fraction (supernatant after centrifugation at 3600 × g for 5 min) and the amount of GS protein detected in the pellet was similar between these two genotypes (data not shown). We confirmed there was no compensatory expression of the liver GS isoform in tissues of $GS^{R582A/R582A}$ mice, as it was expressed at a similar level in the liver of wild-type and $GS^{R582A/R582A}$ animals and was undetectable in muscles in both wild-type and $GS^{R582A/R582A}$ mice (Figure 2D). We next tested the effect of G6P on GS activity in wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ mice. As shown in Figure 2E, although G6P robustly activated GS in muscle extracts derived from wild-type animals, there was a complete loss of GS activation by G6P at submaximal (0.3 mM) and saturating (12 mM) concentrations in extracts from $GS^{R582A/R582A}$ mice. G6P-stimulated GS activity in muscle lysates from $GS^{+/R582A}$ animals was reduced by more than 50% compared to wild-type (~75%), suggesting that the mutant enzyme exerts a dominant-negative effect on wild-type GS. The G6P-independent activity (0 mM) was comparable in all three genotypes.

Insulin Signaling and Phospho-Dependent GS Activation Are Normal in $GS^{R582A/R582A}$ Mice

We next examined the regulation of phospho-dependent GS activity by insulin in $GS^{R582A/R582A}$ knockin animals. Insulin injection robustly stimulated PKB phosphorylation and activity to the same extent in wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ animals (Figures 3A and 3B). PKB reduces the activity of GSK3α/β by phosphorylation of inhibitory serine residues (S21/S9), which was induced by insulin to a similar extent across all three genotypes (Figure 3B). Using phospho-specific antibodies directed against GSK3-target sites on GS (Ser641/Ser645), we found that insulin promoted a marked dephosphorylation that was similar in the muscles of the wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ animals (Figure 3D). There was no significant difference in phospho-dependent GS activity (measured in the absence of G6P) for the three genotypes, and insulin injection resulted in an ~2-fold activation (Figure 3C). These results demonstrate that insulin-mediated phospho-dependent GS activation is normally maintained in GS knockin mice.

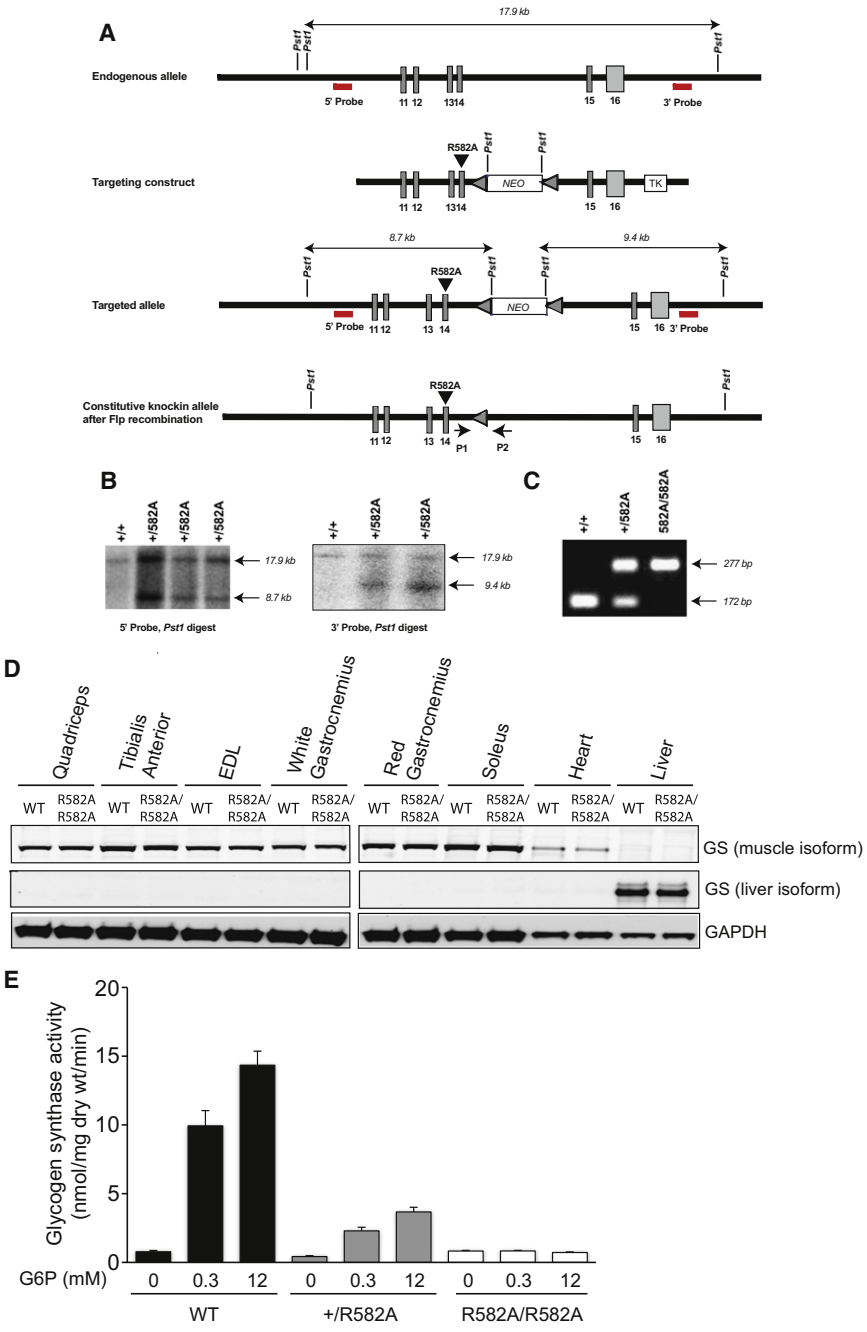

Figure 2. Targeting Strategy Employed to Generate *GYS1* R582A Knockin Mutation and Tissue Expression and Activity of GS in $GS^{R582A/R582A}$ Knockin and Wild-Type Mice

(A) Diagram showing the endogenous *GYS1* allele, the targeting knockin construct, the targeted allele with the neomycin selection cassette (NEO) still present, and the targeted allele with the NEO removed by Flp recombinase. The gray boxes represent exons (6–11), and the gray triangles represent the *FRT* sites. The knockin allele containing the Arg582Ala mutation in exon 14 is illustrated as a black rectangle.

(B) Genomic DNA isolated from the targeted embryonic stem cells from the indicated genotypes was digested with PstI and subjected to Southern blot analysis with the corresponding DNA probes (red boxes). The endogenous wild-type allele generates a 17.9 kb fragment with both 5′ and 3′ probes, while the targeted knockin allele gives rise to a 8.7 kb fragment with the 5′ probe (left panel) and a 9.4 kb fragment with the 3′ probe (right panel).

(C) Genomic DNA isolated from mouse ear biopsies was subjected to PCR analysis with the primer pairs, P1 and P2. The wild-type allele produces a 172 bp DNA fragment, while the knockin allele produces a 277 bp fragment.

(D) Equal amounts of extracts from skeletal muscle (various), heart, and liver isolated from the indicated genotypes were immunoblotted with total muscle or total liver GS isoform-specific antibodies. GAPDH was immunoblotted as a loading control. Results are representative of two independent experiments performed with tissues from four mice.

(E) Gastrocnemius muscle from the indicated genotypes was removed and freeze dried, and extracts were prepared. GS activity was measured in the absence and presence of a range of G6P concentrations. Results are representative of two independent experiments (n = 8/group).

Allosteric Activation of GS Plays a Major Role in Glycogen Synthesis In Vivo

We next sought to determine the role that allosteric activation of GS by G6P plays in muscle glycogen synthesis. The rate of glycogen synthesis in isolated skeletal muscle was measured by monitoring incorporation of [^{14}C]-glucose into endogenous glycogen particles. Soleus muscle, containing predominantly slow twitch-oxidative fibers, or extensor digitorum longus (EDL) muscle, containing predominantly fast twitch-glycolytic fibers, was isolated and incubated in the presence of 5.5 mM glucose with or without insulin. In the absence of insulin, glycogen synthesis in both muscle types was reduced by ~20%–30% in $GS^{+/R582A}$ and ~70% in the $GS^{R582A/R582A}$ mice when compared to wild-type animals (Figures 4A and 4B). Insulin stimulated glycogen synthesis by 4- and 2-fold in soleus (Figure 4A) and EDL (Figure 4B), respectively, which was reduced ~50% in $GS^{+/R582A}$ mice and by ~80% in both muscle types isolated from $GS^{R582A/R582A}$ mice compared to wild-type. We next measured glycogen concentration in muscle tissue. We found a robust decrease (~50%) and an intermediate (~20%–30%) reduction in glycogen content in muscle derived from $GS^{R582A/R582A}$ and $GS^{+/R582A}$ knockin mice, respectively, under both fasted and fed conditions (Figure 4C and Figures S2A and S2B). We observed that the levels of liver glycogen were comparable between wild-type and knockin animals in fasted and fed states (Figure 4D). In order to check if an enhanced rate of glycogen degradation contributed to the reduced muscle glycogen content observed in $GS^{+/R582A}$ and $GS^{R582A/R582A}$

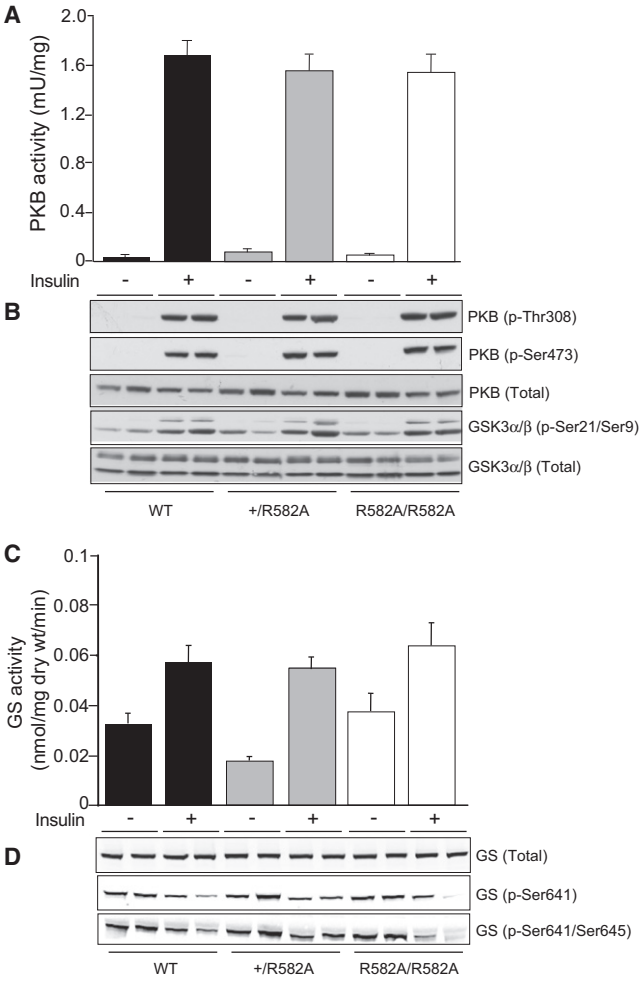

Figure 3. Analysis of Insulin Signaling and Phospho-Dependent GS Activity in GS$^{R582A/R582A}$ Mice and Wild-Type Mice

The indicated mice were fasted overnight and injected intraperitoneally with insulin or vehicle (saline) under anesthesia. After 40 min, gastrocnemius muscle was isolated, and tissue homogenates were assayed as follows.

(A) PKB kinase activity was measured in immunoprecipitates as described in the Supplemental Experimental Procedures. The assay was performed in duplicate (n = 4–6/group).

(B) PKB and GSK3α/β expression and phosphorylation were assessed by immunoblotting with the indicated antibodies. Results are representative of two independent experiments performed with tissues from four mice.

(C) GS activity in the absence of G6P was assayed. The assay was performed in duplicate and the data shown are representative of two independent experiments (n = 4–6/group).

(D) Total and phosphorylated GS were assessed by immunoblotting with the indicated antibodies.

knockin mice, we measured the activity of glycogen phosphorylase, a rate-limiting enzyme in glycogen breakdown. Phosphorylase activity in resting and insulin-stimulated muscles was similar between wild-type and GS knockin mice (Figure 4E). Consistent with this observation, phosphorylation at Ser15, a key regulatory residue, was unchanged in the different genotypes in both basal and insulin-stimulated states (Figure 4E).

To determine if the residual increase in muscle glycogen synthesis in response to insulin observed in GS$^{R582A/R582A}$ mice is due to dephosphorylation of GS via GSK3 inactivation, we have generated triple knockin mice that carry mutations in GS (GS$^{R582A/R582A}$) and GSK3α and -β (GSK3α/β$^{S21A/S21A/S9A/S9A}$). The triple knockin mice were viable, of normal size, and exhibited no overt phenotype. Immunoblot analysis showed that insulin failed to promote significant dephosphorylation of GS at Ser641 in muscles from the triple knockin mice (Figure S2E), consistent with our previous findings in GSK3α/β$^{S21A/S21A/S9A/S9A}$ mice (McManus et al., 2005). These mice displayed an additional ~10% decrease in insulin-stimulated glycogen synthesis compared to GS$^{R582A/R582A}$ mice in insulin-stimulated glycogen synthesis compared to GS$^{R582A/R582A}$ mice, although there remained a significant increase in glycogen synthesis upon insulin treatment (Figure 4F).

Reduced Glycogenic Activity Results in an Increased Glucose Flux through the Glycolytic Pathway

To investigate if the reduction in glycogen synthesis observed in muscles from GS$^{R582A/R582A}$ mice was due in part to impaired glucose transport activity, we measured 2-deoxyglucose uptake in isolated muscles ex vivo. We observed no difference in resting glucose uptake between wild-type and GS knockin animals, and insulin stimulated glucose uptake by ~4-fold and ~1.5-fold in soleus (Figure 5A) and EDL (Figure 5B), respectively, in all genotypes. Immunoblot analysis confirmed that neither GLUT1 nor GLUT4 protein levels were altered in muscles from GS$^{R582A/R582A}$ mice (Figure 5G). Resting as well as insulin-stimulated G6P levels in soleus muscles from GS$^{R582A/R582A}$ mice were higher (~2- and ~2.5-fold, respectively) than those from wild-type mice (Figure 5C). UDP-glucose levels in resting muscles were not significantly different between GS$^{R582A/R582A}$ and wild-type mice, whereas insulin robustly reduced (2-fold) UDP-glucose levels in the soleus of wild-type mice alone and was without effect on GS$^{R582A/R582A}$ animals (Figure 5D). We measured the levels of hexokinase II and UDP-glucose pyrophosphorylase in muscle extracts and confirmed that expression of both enzymes was unchanged in the muscle of GS$^{R582A/R582A}$ mice (Figure 5G). Since glycogen synthesis was profoundly reduced in the muscles of GS$^{R582A/R582A}$ animals, we hypothesized that glycolytic utilization of glucose would be enhanced in these muscles. Soleus muscle was incubated in the presence of 5.5 mM glucose containing [5-^{3}H]-glucose in KRB with or without insulin. The rate of glycolysis was monitored by measuring [^{3}H] H_2O released from the muscle into the incubation medium, and glycogen synthesis was assessed by monitoring [5-^{3}H] glucose incorporation into glycogen (Figure 5E). The rate of glycolysis in both resting and insulin-stimulated muscles was robustly increased (~2.5-fold) in GS$^{R582A/R582A}$ mice relative to that of wild-type animals. Consistent with this observation, lactate release was also higher in GS$^{R582A/R582A}$ muscle in both basal and insulin-stimulated conditions relative to wild-type muscle (Figure 5F).

GS$^{R582A/R582A}$ Mice Display Normal Whole-Body Glucose Disposal and Insulin Sensitivity

We investigated if reduced muscle glycogen synthesis and elevated intramuscular G6P levels would affect whole-body glucose homeostasis in GS$^{R582A/R582A}$ knockin mice. As illustrated in Figures 6A and 6B, there was no difference in blood glucose and plasma insulin levels from overnight fasted or

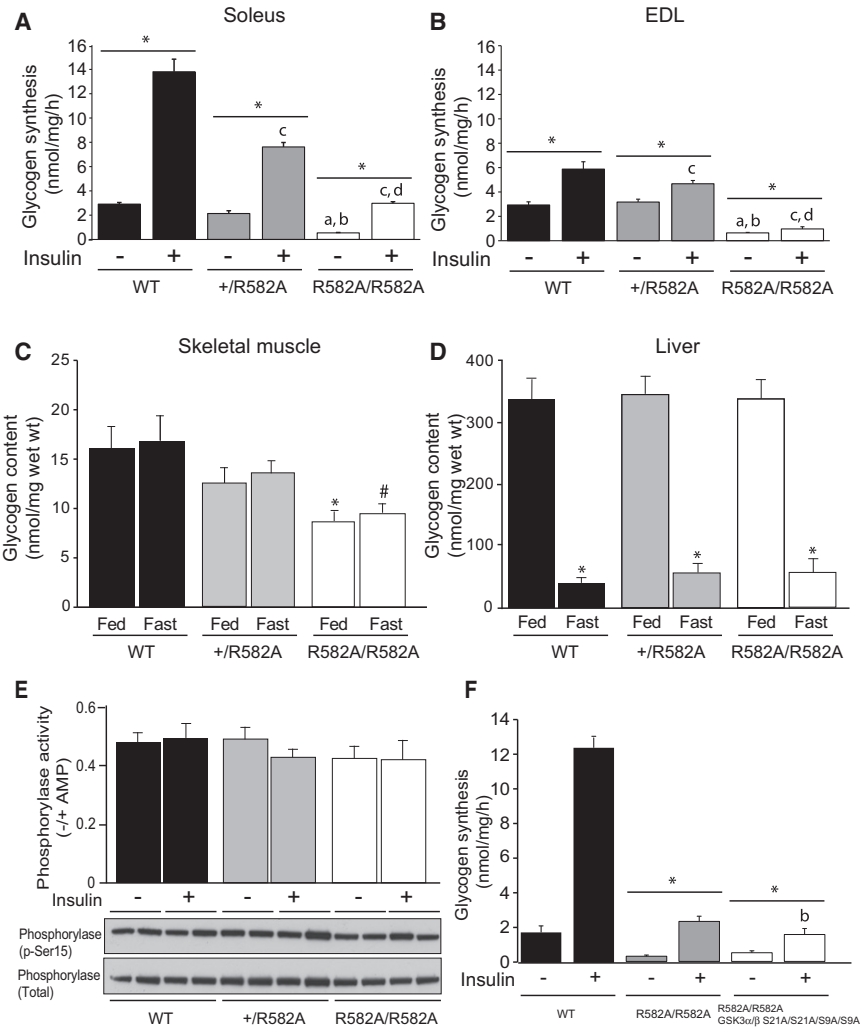

Figure 4. Analysis of Muscle Glycogen Metabolism in GS$^{R582A/R582A}$ Knockin and Wild-Type Mice

(A–D) Soleus or EDL muscles were isolated from the indicated mice following overnight fast and incubated in KRB buffer in the presence or absence of 100 nM insulin. Glycogen synthesis in soleus (A) and EDL (B) was measured as described in the Supplemental Information. *p < 0.05 basal versus insulin within each genotype; (a) p < 0.05 versus WT (basal); (b) p < 0.05 versus +/R582A (basal); (c) p < 0.05 versus WT (insulin); (d) p < 0.05 versus +/R582A (insulin) (n = 5–7/group). (C and D) The indicated mice were fasted overnight or fed ad libitum and muscle (gastrocnemius, C) and liver (D) glycogen content was assessed. *p < 0.05 WT (Fast) versus other genotypes (Fed); #p < 0.05 WT (Fed) versus other genotypes (Fed). (n = 5–7/group) (see also Figure S2).

(E) The indicated mice were fasted overnight and injected intraperitoneally with insulin or saline (basal) under anesthesia. After 40 min, gastrocnemius muscle was removed, snap frozen, and processed to generate tissue extracts. Phosphorylase activity was measured as described in the Supplemental Experimental Procedures. Expression and phosphorylation of phosphorylase were assessed by immunoblotting muscle lysates with the indicated antibodies. (n = 4–5/group).

(F) The rate of glycogen synthesis in resting and insulin-stimulated EDL muscles from the indicated genotypes was measured ex vivo. Statistical analysis (ANOVA) was performed to reveal the difference between GS$^{R582A/R582A}$ and triple knockin animals. *p < 0.05 basal versus insulin within each genotype; (b) p < 0.05 versus GS$^{R582A/R582A}$ (insulin). (n = 6–8/group).

randomly fed animals between wild-type and GS$^{R582A/R582A}$ knockin mice. We next performed glucose tolerance tests and found that GS$^{R582A/R582A}$ knockin mice were able to dispose of injected glucose at the same rate compared to their wild-type littermates in both males (Figure 6C) and females (data not shown). Similarly, insulin tolerance tests revealed an identical profile of insulin-induced hypoglycemia and recovery over the time period tested in wild-type and GS$^{R582A/R582A}$ knockin mice (Figure 6D). To further investigate the insulin sensitivity of GS$^{R582A/R582A}$ knockin mice in a more physiological context, we performed euglycaemic-hyperinsulinaemic clamps, a method that is considered the gold standard for assessing whole body glucose homeostasis in vivo. GS$^{R582A/R582A}$ knockin mice displayed no modification in whole-body glucose metabolism judged by similar rates of glucose infusion and disappearance during the insulin clamp (Figures 6E and 6F and Figure S3).

DISCUSSION

GS is an enzyme of historical importance, as it was the first example of an intracellular target for insulin (Villar-Palasi and Larner, 1960) and is also classically used as an example of enzymes that are controlled via allosteric and covalent mechanisms in cells. Kinetic properties of the differentially phosphorylated forms of GS in cell-free assays have been extensively documented since 1959 (Leloir et al., 1959; Roach, 2002), and several transgenic mouse studies suggest physiological significance of both allosteric and covalent regulation of GS in glycogen accumulation in intact skeletal muscle tissue (reviewed in Lawrence and Roach, 1997). However, the relative importance of these two regulatory mechanisms, particularly the contribution of allosteric regulation to the control of GS and glycogen storage in muscle, is not established, mainly due to the complex interplay between multiple phosphorylation sites and allosteric effectors and the absence of robust experimental tools (e.g., cell-based assays and genetic animal models). Identification of key residues involved in the allosteric regulation of GS by G6P (Figure 1 and Pederson et al., 2000) and generation of the GS$^{R582A/R582A}$ knockin mouse enabled us to overcome various inherent problems (discussed in Bouskila et al., 2008), and here we provide compelling evidence that allosteric activation of GS is the primary mechanism by which insulin promotes

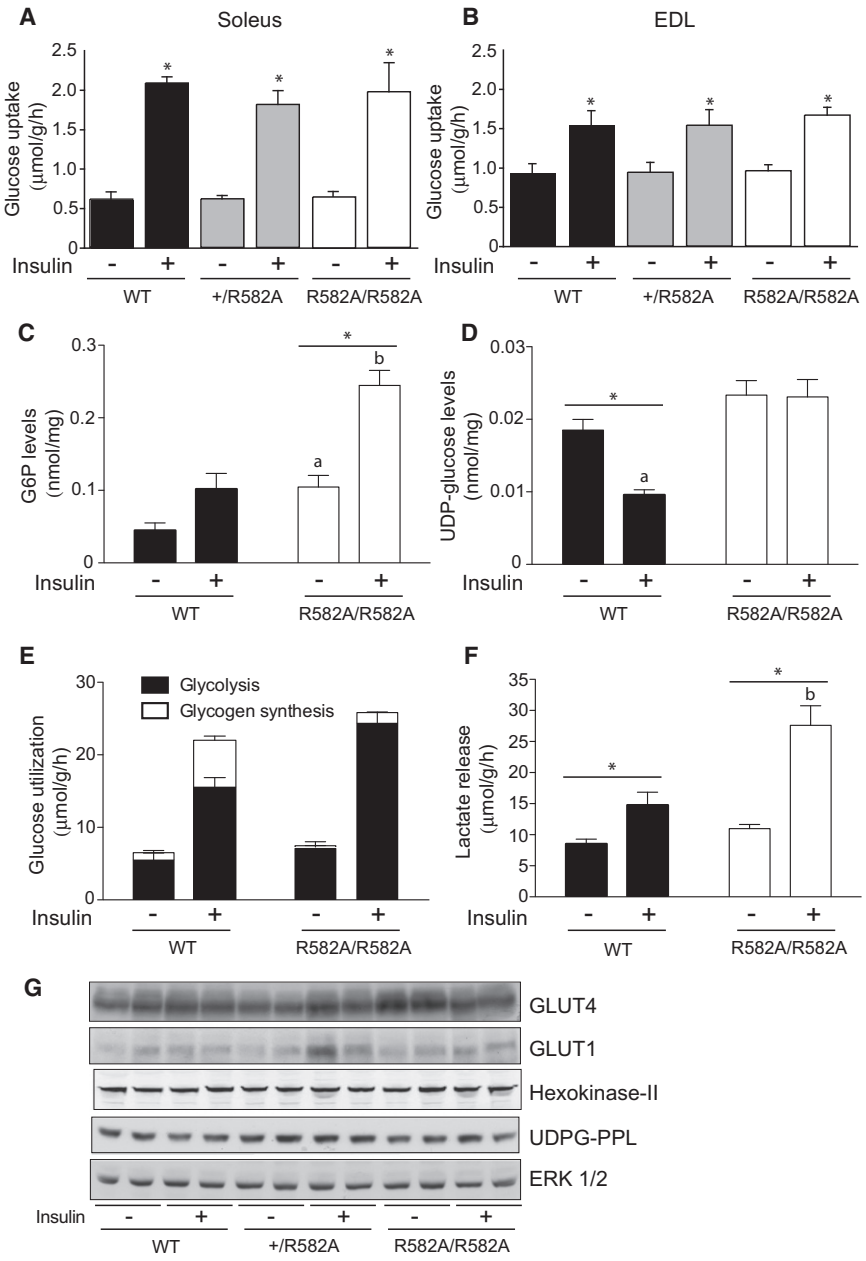

Figure 5. Analysis of Muscle Glucose Utilization in GS$^{R582A/R582A}$ Knockin and Wild-Type Mice

Soleus or EDL muscles were isolated from the indicated genotypes following overnight fast and incubated in KRB buffer in the presence or absence of 100 nM insulin.

(A and B) 2-deoxyglucose uptake in soleus (A) or EDL (B) was measured as described in the Supplemental Experimental Procedures. *p < 0.05 basal versus insulin within each genotype; (n = 5–7/group).

(C–E) Isolated soleus muscle was incubated in KRB buffer containing 5.5 mM glucose for 40 min with or without 100 nM insulin. Muscle G6P (C) and UDP-glucose (D) levels and lactate release (E) were measured as described in the Supplemental Information.

(F) Isolated soleus was incubated in KRB buffer with 5.5 mM glucose containing [5-^{3}H]-glucose for 40 min in the presence or absence of 100 nM insulin. Glucose utilization by glycolysis and glycogenesis was assessed by measuring [^{3}H]-H_2O accumulation in the incubation buffer and [^{3}H]-glucose incorporation into glycogen, respectively, as described in the Experimental Procedures. *p < 0.05 basal versus insulin within each genotype; (a) p < 0.05 versus WT (basal); (b) p < 0.05 versus WT (insulin). (n = 3–4/group).

(G) The indicated mice were fasted overnight, and gastrocnemius muscles from saline- or insulin-injected (i.p., 150 mU/g body weight, 40 min) animals were removed and tissue extracts generated. The expression of GLUT1, GLUT4, hexokinase II, and UDP-glucose pyrophosphorylase (UDPG-PPL) was assessed in muscle extracts by immunoblotting with the indicated antibodies. Four to five samples for each condition from the indicated mice were analyzed, and representative blots are shown.

glycogen synthesis upon incorporation of glucose into muscle cells. Analysis of triple GS$^{R582A/R582A}$/GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ knockin mice, in which both allosteric- and phospho-dependent activation of GS by insulin is prevented, complemented the results obtained from individual GS$^{R582A/R582A}$ (current study) and GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ (Bouskila et al., 2008; McManus et al., 2005) knockin mice. We observed a modest, but significant decrease (~10%) in insulin-stimulated glycogen synthesis in the muscle of the triple GS$^{R582A/R582A}$/GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ knockin compared to that of GS$^{R582A/R582A}$ mice (Figure 4F). Therefore, dephosphorylation of GS as occurs with insulin is likely to play a "fine-tuning" role for GS by promoting changes in enzyme kinetic properties, which would be expected to enhance the affinity for the substrate UDP-glucose and also sensitivity to G6P (Friedman and Larner, 1963; Jensen and Lai, 2009). Whether G6P-bound/dephosphorylated GS proteins change localization for efficient glycogen formation would be an interesting subject to explore in the future. The mechanism underlying the residual increase in glycogen synthesis in response to insulin in triple GS$^{R582A/R582A}$/GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ knockin mice is unclear. There was no detectable expression of the liver GS isoform (GYS2) in the muscle of GS$^{R582A/R582A}$ mice (Figure 2D), and it is unlikely that an alternative GS-like enzyme catalyzes the formation of glycogen, as muscle GS-deficient animals display a loss of glycogen (Pederson et al., 2004). Although it would be reasonable to assume that elevated UDP-glucose arising from elevated insulin-stimulated glucose uptake would increase glycogen synthesis by mass action, results from our work and others are not sufficient to draw this conclusion. An increase in the levels of G6P would be expected to increase concentrations of downstream intermediates by mass action in the glycogen

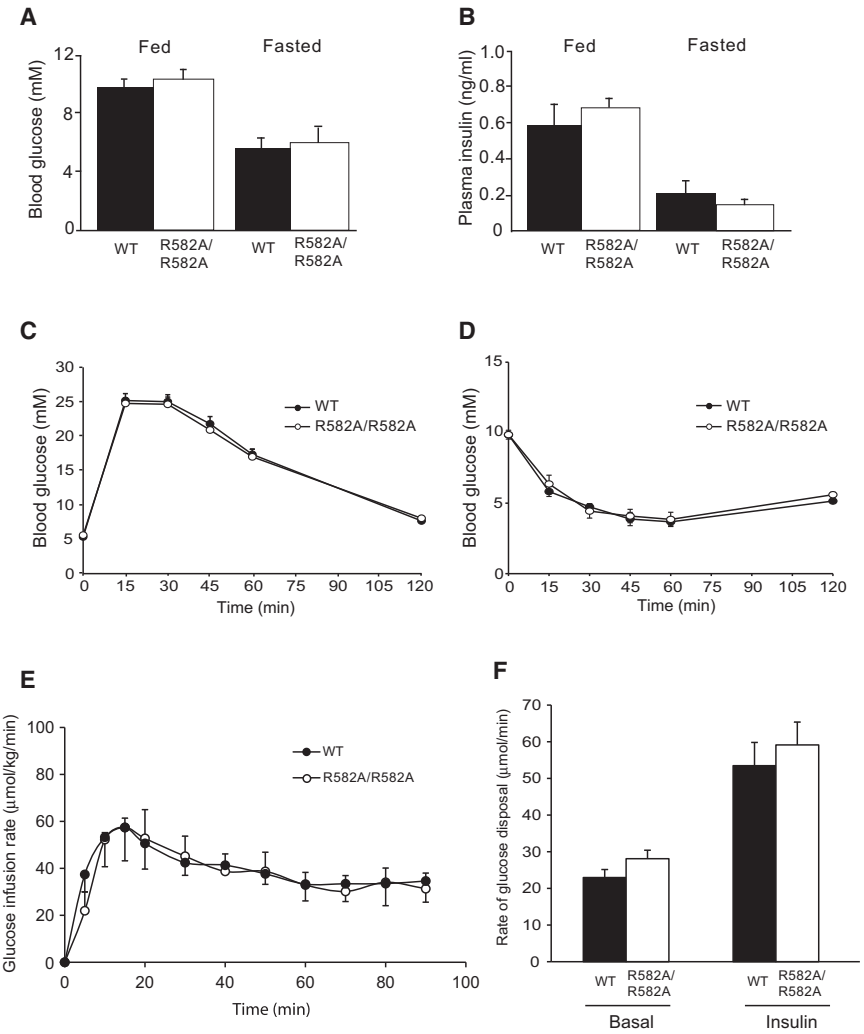

Figure 6. Glucose Tolerance, Insulin Sensitivity, and Plasma Insulin Levels in $GS^{R582A/R582A}$ Knockin and Wild-Type Mice

(A and B) Approximately equal numbers of 2- to 3-month-old male and female mice from indicated genotypes (n = 6–7 animals) were fasted overnight (Fasted) or fed ad libitum (Fed), and blood glucose and plasma insulin levels were determined.

(C and D) Blood glucose concentration in 2- to 3-month-old male $GS^{R582A/R582A}$ knockin and wild-type mice was measured at the indicated times following intraperitoneal administration of 2 mg/g glucose following overnight fast or 0.75 mU/g intraperitoneal insulin injection following 4 hr food removal (n = 7–9 animals/genotype).

(E and F) A euglycaemic-hyperinsulinaemic clamp study was performed as described in the Supplemental Experimental Procedures using four male mice from each genotype (~4 months old). The clamp study started with a bolus of insulin (100 mU/kg), followed by continuous insulin infusion (3.5 mU/kg/min). A variable amount of 12.5% glucose solution was infused to maintain euglycaemia (~5 mmol/l) (E and Figure S3) during a 90 min clamp, and whole-body glucose disposal prior to and at the end of insulin infusion was calculated (F) (n = 4 animals/genotype). Steady-state-specific activities for ^{14}C-glucose during both the basal and hyperinsulinaemic period were achieved (data not shown).

synthesis pathway. In contrast, we (Figure 5D) and others (Reynolds et al., 2005) observed that UDP-glucose levels were reduced in muscles from wild-type animals with insulin, most likely because a sufficient increase in G6P as well as insulin-mediated dephosphorylation would activate GS, thereby elevating the rate of UDP-glucose utilization. Our finding that there was no decrease in UDP-glucose levels with insulin in the muscles of $GS^{R582A/R582A}$ mice correlates with the markedly reduced activity of muscle GS in these animals. Of note, a previous work reported that the overexpression of UDP-glucose pyrophosphorylase and an associated increase in the levels of UDP-glucose do not affect glycogen synthesis in resting and insulin-stimulated mouse skeletal muscles (Reynolds et al., 2005). Finally, although it was not significant, there was a trend for a reduction in GS phosphorylation (Ser641) in muscles from the triple knockin mice in response to insulin (Figure S2E), and we cannot completely rule out the possibility that insulin caused GSK3-independent activation of GS (e.g., PP1-dependent) as a compensatory mechanism.

We observed some dissociation between the reduction of glycogen synthesis (~70%–80%) measured in resting and insulin-stimulated muscles ex vivo (Figures 4A and 4B) and steady-state muscle glycogen content (~50%) in overnight fasted or randomly fed $GS^{R582A/R582A}$ mice compared to those of wild-type animals (Figure 4C). There are several possible explanations. First, it is possible that insulin-independent activation of GS contributes to glycogen synthesis and maintains glycogen levels in skeletal muscle in vivo. It is well known that muscle contraction robustly stimulates GS in order to rapidly restore glycogen content following its breakdown by the action of phosphorylase for energy supply. This occurs via dephosphorylation of GS, but in a PKB/GSK3-independent (McManus et al., 2005) and PP1-dependent manner (Aschenbach et al., 2001). Second, a reduced capacity to synthesize muscle glycogen in GS knockin mice may have led to metabolic adaptations resulting in the muscles of these animals being more efficient at utilizing extracellular glucose and/or fatty acids as substrates for energy production during physical activity, producing a glycogen sparing effect (Pederson et al., 2005). Interestingly, we observed a shift of glucose utilization toward glycolysis in the muscles of GS knockin animals (Figures 5E and 5F). It would be of interest to investigate if oxidation of glucose and fatty acids is increased in muscles of $GS^{R582A/R582A}$ mice during exercise. Third, given that steady-state levels of glycogen are determined by the balance between synthesis and degradation in vivo, it is possible that glycogenolysis was inhibited as a result of some compensatory mechanism in muscles of $GS^{R582A/R582A}$ knockin mice to avoid a profound

depletion of glycogen. We showed that the activity of glycogen phosphorylase in muscle lysate was similar between the GS knockin and wild-type animals (Figure 4E). However, like GS, muscle phosphorylase activity is coordinately regulated by phosphorylation and allosteric stimulators (e.g., AMP) and inhibitors (e.g., ATP, G6P) (Barford et al., 1991), and the cell-free assay is largely a measure of the phospho-dependent activity. Therefore, we cannot rule out the possibility that elevated levels of G6P allosterically inhibited muscle phosphorylase in vivo. It is unlikely that cellular levels of AMP and ATP were altered in resting muscle of GS knockin mice, as activity of the AMP-activated protein kinase, which is regulated by changes in AMP:ATP ratio (Hardie and Sakamoto, 2006), was unchanged (Figures S2C and S2D). To investigate the inhibitory effect of G6P on muscle phosphorylase and glycogen metabolism in vivo, it will be necessary to generate G6P-insensitive phosphorylase knockin animals.

Glycogen synthesis is considered to be of major importance for glucose homeostasis, as muscle with an impaired ability to synthesize glycogen is proposed to attenuate its ability to remove glucose from the circulation, thereby causing insulin resistance and type 2 diabetes. This is based on the assumption that impaired glycogenic activity would be expected to promote accumulation of intracellular G6P levels, which led to the inhibition of hexokinase, thereby reducing the rate of glucose transport (Shulman, 2000). Consistent with this notion, we observed that G6P levels are significantly elevated in muscle from GS knockin compared to that from wild-type mice (Figure 5C). We showed that resting as well as insulin-stimulated glucose transport, assessed by [^{3}H]-2-deoxyglucose uptake, was comparable in isolated soleus and EDL muscles from GS knockin relative to those of wild-type animals (Figure 5A). Although this confirmed that there was no impairment in glucose transport activity in the GS knockin muscles, whether elevated levels of G6P would affect glucose transport under more physiological conditions (e.g., 5–8 mM glucose) could not be addressed under these experimental conditions (no glucose and 1 mM 2-deoxyglucose in the transport buffer). Although 2-deoxyglucose uptake accurately reflects glucose transport activity (Hansen et al., 1994), its intracellular accumulation does not promote feedback inhibition of muscle hexokinase. A previous study showed that in cell-free assays, G6P inhibits hexokinase by 80% at a concentration of 0.5 mM in muscle homogenates, whereas even at a concentration of 30 mM, 2-deoxyglucose-6-phosphate fails to inhibit the enzyme by 80% (Hansen et al., 1994). However, it should be noted that when isolated soleus was incubated under euglycaemic conditions (5.5 mM glucose) ex vivo, glucose utilization, as judged by the sum of glycogen synthesis and glycolysis, was not reduced in knockin muscles (Figure 5E), suggesting that glucose transport was not significantly inhibited. Whether sustained accumulation of G6P occurs under physiological conditions (e.g., following glucose ingestion) in vivo would be interesting, and kinetic analysis of muscle G6P using noninvasive nuclear magnetic resonance analysis can potentially shed light on this. Nonetheless, our GS$^{R582A/R582A}$ knockin mouse model provides a unique opportunity to test if reduced glycogenic activity impairs whole-body glucose homeostasis. We demonstrate that a drastically reduced rate of muscle glycogen synthesis does not necessarily cause impaired whole-body glucose disposal. This is at least in part consistent with a study employing muscle GS-deficient mice that lack glycogen in skeletal and cardiac muscles (Pederson et al., 2005). These mice showed normal blood glucose levels in fasted and fed states and of note they disposed of glucose more effectively than wild-type animals. The mechanism by which GS null mice showed improved clearance of blood glucose was not clear, although the authors speculated that it might be due to sustained elevation of serum insulin in the GS-deficient animals during glucose tolerance tests. The GS null mice were leaner and partially protected against the insulin resistance induced by high-fat-diet feeding. This could be explained by the fact that GS knockout mice had a higher proportion of oxidative fibers with increased phosphorylation of AMPK and ACC, which indicated enhanced capacity in the muscle of GS-deficient mice for more efficient fatty acid and glucose oxidation. As described above, AMPK activity and ACC phosphorylation were not altered in resting muscles between wild-type and GS knockin mice (Figures S2C and S2D). Of note, whole-body insulin sensitivity of GS null mice, judged by glucose infusion rate during a euglycaemic-hyperinsulinaemic clamp, was no different from wild-type animals. Insulin-induced glucose uptake into peripheral tissues was decreased, whereas liver glycogen accumulation was enhanced in the GS knockout mice, indicating muscle insulin resistance. Results from the above study indicate that ablation of muscle GS and a lack of glycogen produce complex metabolic adaptations in various organs to maintain glucose homeostasis. Moreover, the data need to be cautiously interpreted, as the GS deficiency resulted in a cardiac developmental problem, and only ~10% of the GS null mice survive birth (Pederson et al., 2004). Because ~90% of muscle GS knockout mice die shortly after birth, one cannot rule out the possibility that the subset of animals studied was adapted to dispose glucose normally because of the presence of some factor that enabled their survival (Pederson et al., 2005).

Since a reduced capacity to synthesize muscle glycogen did not impair glucose disposal, one might question the importance of muscle glycogen accumulation for blood glucose homeostasis at least in the mouse and also wonder whether mice are an appropriate model to study human metabolism. When expressed as a percentage of total body glycogen, glycogen content in mouse skeletal muscle is only 10% of that in human muscle (Kasuga et al., 2003). In addition, humans have 3- to 8-fold more muscle glycogen than that in liver, whereas fed mice contain five to ten times more total glycogen in liver than in skeletal muscle (Irimia et al., 2010). Therefore, the relative contribution of muscle and hepatic glycogen in controlling glucose homeostasis might be different between mice and humans with hepatic energy reserves of greater importance in mice. Interestingly, some recent reports describing the clinical and metabolic phenotypes of human subjects with mutations/polymorphisms in genes involved in glycogen metabolism suggest a critical role for muscle glycogen accumulation in energy production during exercise, but not for glucose homeostasis. Kollberg et al. have reported that human subjects carrying a homozygous stop mutation in exon 11 in the *GYS1* gene, which is predicted to cause truncation of GS at Arg462, resulted in a loss of GS proteins in both skeletal and heart muscles (Kollberg et al., 2007). Muscle GS-deficient subjects exhibit reduced

exercise capacity with rapid muscle fatigability most likely due to an inability to utilize glycogen as an energy source for ATP generation during muscle contraction. However, they display a normal blood glucose profile when challenged with an oral glucose load. In addition, muscle glycogen depletion due to inactivation of glycogenin-1, caused by biallelic mutations in *GYG1*, resulted in muscle weakness and cardiac arrhythmia; however, the patient exhibited normal glucose tolerance, and levels of fasting glucose and glycated haemoglobin were also normal (Moslemi et al., 2010). Taking our current results and previous findings together, it can be argued that impaired glycogen synthesis as seen in type 2 diabetics may not be a primary cause of insulin resistance; rather it is a consequence due to multiple defects in the insulin signaling pathway, resulting in impaired glucose transport (Shulman, 2000). A reduced rate of glucose transport and concomitant decrease in intramuscular accumulation of G6P would attenuate glycogen synthesis via reduced allosteric activation of GS. Therefore, glucose transport is the critical step for insulin-stimulated glycogen synthesis, and suggests that muscle glucose transport represents a more important therapeutic target for insulin resistance and type 2 diabetes.

We propose that increased levels of intramuscular G6P via glucose transport in response to insulin allosterically stimulate GS, which drives it into glycogen as a feed-forward mechanism, and this effect could be further enhanced when GS is dephosphorylated by inactivation of GSK3 (Patel et al., 2008), as this makes GS more sensitive to G6P. This proposition is consistent with the metabolic control theory proposed by Shulman and Rothman (Shulman and Rothman, 1996). The interplay between allosteric regulation and covalent phosphorylation allows GS to detect small changes in the levels of G6P when elevated upon insulin stimulation. This sophisticated enzyme control system facilitates a sustained influx of glucose, which would prevent a buildup of the levels of intermediates. There are many examples of metabolic enzymes that are coordinately regulated via an interplay between allostery and phosphorylation, and elucidating such interplay would be fundamental in understanding the in vivo regulation of metabolic flux control.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Transfection, and Purification of GS

HEK293 cells were cultured using standard protocols and transfected with 20 μl of polyethylenimine (1 mg/ml) and 5–10 μg of plasmid DNAs per 10 cm dish. Thirty-six hours following transfection, cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer. Lysates were clarified by centrifugation and the supernatant assayed for protein concentration using Bradford reagent. Cell extracts (~70–100 mg protein) generated from cotransfection of untagged GS (wild-type or mutants) and GST-tagged glycogenin were incubated with glutathione Sepharose for 1 hr at 4°C and the resin washed twice with lysis buffer containing 0.5 M NaCl and twice with buffer A (50 mM Tris-HCl [pH 7.5], 0.1 mM EGTA). GST-glycogenin:GS complexes were eluted with 20 mM reduced glutathione. The eluted proteins and BSA standards were resolved on SDS-PAGE and stained with Coomassie blue R250. The band corresponding to GS was scanned and the protein concentration estimated by densitometric analysis using a LI-COR Odyssey imaging system.

Glycogen Synthase Activity

GS activity was measured by a modification of the method of Thomas et al. (Thomas et al., 1968) and described in detail (Lai et al., 2007). Muscles were freeze dried for 3 hr, and 2–3 mg tissue (dry weight) was homogenized (1:400; dry tissue weight:volume) with a rotor-stator homogenizer (Polytron, Kinematica AG) in ice-cold buffer containing 50 mM Tris-HCl (pH 7.8), 100 mM NaF, 10 mM EDTA. Homogenates were centrifuged for 5 min at 3600 × g at 4°C and GS activity measured in supernatants as follows: 20 μl of supernatant was added to 40 μl assay buffer (25 mM Tris-HCl [pH 7.8], 50 mM NaF, 5 mM EDTA, 10 mg/ml glycogen, 1.5 mM UDP-glucose, and 0.5 μCi/ml D-[^{14}C]-UDP-glucose) with 0, 0.3, and 12 mM G6P. Reactions were incubated for 8 min at 37°C and stopped by spotting 50 μl onto squares of filter paper (Whatman ET-31) which were immediately immersed in ice-cold 66% ethanol and washed twice more in 66% ethanol. Dried filters were subjected to scintillation counting.

Determination of Muscle Glucose Utilization

Muscle glycolytic rates were determined by the detritiation of [5-^{3}H]-glucose during the reactions catalyzed by triose phosphate isomerase and enolase. The glucose tracer was dried using a Savant Speedvac concentrator to remove [^{3}H] H_2O accumulated during storage as a consequence of radiolysis. Isolated soleus muscles were incubated in 2 ml Krebs-Ringer bicarbonate (KRB) containing 5.5 mM glucose and 0.5 mCi.mmol [5-^{3}H]-glucose for 40 min at 37°C, gassed continuously with 95%/5% O_2/CO_2. Muscles were snap-frozen in liquid nitrogen and processed for determination of [5-^{3}H] glucose incorporation into glycogen as described for [^{14}C(U)]glucose. [^{3}H] H_2O was isolated by borate complex ion exchange chromatography. Briefly, aliquots (0.5 ml) of conditioned KRB were applied to 1 ml columns of Dowex-1-borate and washed with 2 ml water to elute [^{3}H] H_2O. Glucose forms a borate complex and is retained by the resin. Columns were regenerated with 0.5 M potassium tetraborate. [^{3}H] H_2O was determined by scintillation counting and corrected for leakage of [5-^{3}H]-glucose (typically less than 0.03%).

Statistical Analyses

Data are expressed as mean ± SEM. Statistical analysis was performed by two-tailed Student's t test or one-way ANOVA with Newman-Keuls multiple comparison post hoc test. Differences between groups were considered as statistically significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2010.10.006.

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