Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle

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Bouskila M, Hirshman MF, Jensen J, Goodyear LJ, Sakamoto K. Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. Am J Physiol Endocrinol Metab 294: E28–E35, 2008.—Insulin promotes dephosphorylation and activation of glycogen synthase (GS) by inactivating glycogen synthase kinase (GSK) 3 through phosphorylation. Insulin also promotes glucose uptake and glucose 6-phosphate (G-6-P) production, which allosterically activates GS. The relative importance of these two regulatory mechanisms in the activation of GS in vivo is unknown. The aim of this study was to investigate if dephosphorylation of GS mediated via GSK3 is required for normal glycogen synthesis in skeletal muscle with insulin. We employed GSK3 knockin mice in which wild-type GSK3α and β genes are replaced with mutant forms (GSK3αβ/S21A/S9A/S9A), which are nonresponsive to insulin. Although insulin failed to promote dephosphorylation and activation of GS in GSK3αβ/S21A/S9A/S9A mice, glycogen content in different muscles from these mice was similar compared with wild-type mice. Basal and epinephrine-stimulated activity of muscle glycogen phosphorylase was comparable between wild-type and GSK3 knockin mice. Incubation of isolated soleus muscle in Krebs buffer containing 5.5 mM glucose in the presence or absence of insulin revealed that the levels of G-6-P, the rate of [14C]glucose incorporation into glycogen, and an increase in total glycogen content were similar between wild-type and GSK3 knockin mice. Injection of glucose containing 2-deoxy-[3H]glucose and [14C]glucose also resulted in similar rates of muscle glucose uptake and glycogen synthesis in vivo between wild-type and GSK3 knockin mice. These results suggest that insulin-mediated inhibition of GSK3 is not a rate-limiting step in muscle glycogen synthesis in vivo. This suggests that allosteric regulation of GS by G-6-P may play a key role in insulin-stimulated muscle glycogen synthesis in vivo.

The biosynthesis of glycogen first requires glucose to be transported into muscle cells, and then glucose is phosphorylated by hexokinase to form glucose 6-phosphate (G-6-P). G-6-P can be used for the synthesis of glycogen or metabolized in the glycolytic pathway. In the former case, G-6-P is converted to UDP-glucose, and GS subsequently catalyses the synthesis of glycogen by transferring a glucosyl moiety from UDP-glucose to a preexisting glycogen molecule. GS is regulated by covalent phosphorylation, which inhibits the enzyme, and by the allosteric activator G-6-P (22). The relative contribution of these two events in the activation of muscle GS in vivo is not well understood.

Insulin is thought to enhance muscle GS activity through the activation of protein kinase B (PKB/Akt) and subsequent phosphorylation and deactivation of glycogen synthase kinase 3 (GSK3), which subsequently promotes dephosphorylation of GS at a cluster of COOH-terminal serine residues (4). In support of this, we have recently reported that insulin was unable to promote dephosphorylation of GS at residues targeted by GSK3 (Ser411 and Ser445) and activation of GS in the skeletal muscle of knockin mice expressing constitutively active mutant forms of GSK3 in which the PKB phosphorylation sites on GSKα (Ser21) and GSKβ (Ser22) were changed to Ala (GSKαβ/S21A/S21A/S9A/S9A) (15). However, the levels of glycogen in the quadriceps muscle of either fed or overnight-fasted mice were similar between wild-type and GSKαβ/S21A/S21A/S9A/S9A mice. Because basal and insulin-stimulated muscle glycogen uptake were also similar in muscles of wild-type and GSK3 knockin animals (15), we hypothesized that the similar levels of muscle glycogen in wild-type and GSK3 knockin mice could be explained if activation of GS by the allosteric activator G-6-P plays a key role to synthesize glycogen, compensating for the lack of insulin-induced dephosphorylation of GS in the GSK3αβ/S21A/S21A/S9A/S9A mice. However, given that muscle glycogen content is regulated simultaneously by synthesis and degradation processes, and these events are regulated by several key signaling molecules, it is possible that a lack of insulin-induced dephosphorylation and activation of GS in GSK3 knockin mice resulted in compensatory changes in muscle glycogen breakdown. It has been reported that genetic deletion of the glucose transporter GLUT4 in muscle abolished insulin-stimulated glucose uptake. However, controversially, muscle glycogen content was higher in muscle GLUT4-deficient mice than that of wild-type ani-
mals (11). This was postulated to be because of multiple compensatory changes in glycogen metabolism in muscle GLUT4-null mice, since there was a decrease in glycogen phosphorylase activity, an increase in the expression of hexokinase II, and an elevation of G-6-P level as well as an increase in protein phosphatase-1 activity, and together that would promote an increase in glycogen levels.

The aim of the present study was to investigate whether normal levels of muscle glycogen observed in GSK3 knockin mice, where insulin-induced dephosphorylation and activation of GS through GSK3 cannot occur, was the result of 1) compensatory changes in muscle glycogen metabolism or 2) normal glucose transport and formation of G-6-P that is sufficient to allosterically stimulate GS in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials.** Protease inhibitor cocktail tablets were obtained from Roche (Sussex, UK). Precast SDS-polyacrylamide Bis-Tris gels were from Invitrogen. G-6-P was from Roche. Twenty, 20-g glycogen type III from rabbit liver, UDP-glucose, glucose 1-phosphate, and AMP were from Sigma. α,β-[3H]glucose-1-phosphate, UDP-[1,4-14C]glucose, 2-deoxy-α-[3H]glucose, and α-[14C]glucose were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Human insulin (Novo-Nordisk) and epinephrine were obtained from Ninewells Pharmacy (Dundee, UK). All other chemicals were obtained from Sigma.

**Antibodies.** The total GS antibody was a mouse monoclonal antibody purchased from Chemicon (MAB3106). The phospho-GS antibody (Ser^{11/465}) was raised in sheep against peptide RPYPRPVSPVP-PpSPSLR (corresponding to residues 635–650 of mouse GS). The antibody was affinity-purified on CH-Sepharose covalently coupled to the antigen peptide. The phospho-GS antibody (Ser^{1}) was generated and donated by Professor D. Grahame Hardie (University of Dundee) (10). Anti-phospho-GSK3α/β (Ser^{21/9}) antibody (9331) and anti-phospho-Akt (Thr^{308}) antibody (9275) were purchased from Cell Signaling Technology. Total anti-GSK3α/β was purchased from Biosource (44–610). Anti-hexokinase II antibody was obtained from Santa Cruz Biotechnology (C-6521). Anti-phospho-phosphorylase (Ser^{1}) was raised in sheep against peptide KRKQIPSVRGLA (corresponding to residues 47–60 of human muscle isoform) and affinity purified on CH-Sepharose covalently coupled to the antigen peptide. The antibody was affinity-purified on CH-Sepharose covalently coupled to the antigen peptide. Total anti-phosphorylase antibody was raised in sheep using partially purified phosphorylase protein from rabbit skeletal muscle as antigen. Secondary antibodies were obtained from Pierce or Molecular Probes.

**Animals.** All animal studies were approved by the University of Dundee Ethics Committee and performed under a United Kingdom Home Office project license. GSK3α/β^{21/9} and control wild-type GSK3α/β^{+/+} animals were generated, bred, and geno-typed as previously described (15). Mice (2–6 mo old) were used for the experiments.

**Injection of insulin and epinephrine.** Mice were fasted overnight (~16 h) and anesthetized by an intraperitoneal injection of pentobarbital sodium (75 mg/kg body wt) diluted in PBS. For insulin treatment, 150 μg/kg body wt of insulin diluted in PBS or the same volume of PBS was injected as a bolus intraperitoneally. For epinephrine injection, 0.25 μg/g body wt of epinephrine (10 μg/ml containing 0.88% sodium chloride and sodium metabisulfite; Ninewells Pharmacy) or the same volume of PBS was injected as a bolus intraperitoneally. After insulin or epinephrine injection (40 or 15 min) mice were killed by cervical dislocation, and indicated hindlimbs muscles were removed rapidly and immediately frozen in liquid nitrogen. Muscles were stored at −80°C.

**Preparation of muscle tissue lysates.** Frozen muscle tissues were homogenized (Kinematica Polytron, Brinkmann, CT) in 10-fold mass excess of ice-cold lysis buffer containing 50 mM Tris·HCl, pH 7.5, 2 mM EGTA, 10 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (by volume) 2-mercaptoethanol, and “complete” protease inhibitor cocktail (1 tablet/50 ml). Homogenates were then centrifuged for 5 min at 3,600 g at 4°C, and the supernatant was collected. Total protein concentration was determined by the Bradford method using BSA as standard, and lysates were separated into aliquots, snap-frozen in liquid nitrogen, and stored at −80°C.

**Immunoblotting.** Muscle lysate (20–30 μg) was heated in SDS sample buffer, separated by gel electrophoresis using precast SDS-polyacrylamide Bis-Tris gels, and transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in 50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween 20 (TBST) containing 10% (by mass) skimmed milk or 5% (by mass) BSA and then incubated at 4°C for 16 h with the indicated primary antibodies (1–2 μg/ml for the sheep antibodies and a 1,000-fold dilution for the commercial antibodies were used) in TBST containing 5% (by mass) skimmed milk or 5% (by mass) BSA. Detection was performed either by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent or by fluorescence using a LI-COR Odyssey infrared detection system (http://www.licor.com/) following the manufacturer’s guidelines.

**GS and phosphorylase assays.** GS and phosphorylase activity were determined by measuring incorporation of UDP-[1-14C]glucose into glycogen in the presence or absence of 10 mM G-6-P and the incorporation of [14C]glucose 1-phosphate into glycogen with or without 2 mM AMP, respectively. Activity ratios are the activity measured in the absence divided by that in the presence of the allosteric effectors G-6-P or AMP. Assay procedures were described previously (15, 28).

**Measurement of muscle glycogen content.** Muscle pieces (20–30 mg) were hydrolyzed in 250 μl of 2 N HCl by heating at 95°C for 3 h. The solution was then neutralized with 250 μl 2 N NaOH, and the resulting free glycosyl units were assayed spectrophotometrically using a hexokinase-dependant assay kit from Amresco, as described previously (2).

**Incubation of isolated muscle in vitro.** Mice were fasted overnight (16 h) before experiment and killed by cervical dislocation, and soleus muscles were rapidly and carefully removed. Tendons from both ends of each muscle were tied with suture (silk 4–0) and mounted on an incubation apparatus. The muscles were incubated as previously described (23). Briefly, muscles were incubated in 8 ml of Krebs-Ringer bicarbonate (KRB) buffer (in mM: 117 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, and 24.6 NaHCO3, pH 7.4) containing 5.5 mM d-glucose for 1 or 2 h at 37°C, in the presence or absence of 100 mM insulin. The buffer was continually gassed directly by bubbling with a mixture of 95% O2 and 5% CO2. At the end of the incubation period, muscles were quickly frozen in liquid nitrogen and stored at −80°C. Muscle glycogen content was determined as described above.

**Measurement of glycogen synthesis in vitro.** Isolated muscles were incubated in 2 ml of Krebs-Ringer bicarbonate (KRB) buffer containing 5.5 mM d-glucose and 0.5 μCi/ml of [14C]glucose for 40 min at 37°C in the presence or absence of 100 mM insulin. At the end of the incubation period, muscles were rinsed with ice-cold KRB buffer, blotted on the paper, and quickly frozen in liquid nitrogen. Muscles were weighed and processed as described previously (21). In brief, muscles were digested in 400 μl of 30% KOH at 95°C for 20 min. Glycogen solution (50 μl of a 5% glycogen solution) was added to each sample, and 100% ethanol was added to a final concentration of 70%, and then glycogen was allowed to precipitate at −20°C overnight. The samples were centrifuged at 8,900 g for 20 min at 4°C to pellet glycogen. The glycogen pellets were washed four times with 66% ethanol and dissolved in 50 μl of deionized water. Samples were spotted on 2-cm squares of filter paper.
(Whatman 3MM) that were washed three times with 66% ethanol. The papers were dried before the amount of \(^{14}\text{C}\)glycogen was determined by scintillation counting.

**Measurement of G-6-P concentration.** Muscles were freeze-dried, and visible connective tissues were removed. Muscles were transferred to Eppendorf tubes and precisely weighed. Perchloric acid (PCA, 200 \(\mu\)l of 0.6 M) was added to each tube, and tubes were left on ice for 30 min. After the addition of PCA (15 min), muscles were gently squeezed with a glass pin to extract all metabolites. PCA was neutralized with 65 \(\mu\)l 2 M KHCO\(_3\) and centrifuged for 10 min at 3,000 \(g\) at 4°C, and the supernatant was transferred to a new tube. G-6-P concentration was determined fluorometrically as described (7). Briefly, 100 \(\mu\)l of neutralized extract were added to 600 \(\mu\)l assay buffer containing 50 mM Tris-HCl, pH 8.1, 30 \(\mu\)M NADP\(^+\), and 100 \(\mu\)M dithiothreitol, and blank reading was performed. G-6-P dehydrogenase (5 \(\mu\)l of 7 U/ml; 127,655; diluted in 20 mM Tris, pH 8.1, containing 0.02% BSA; Roche) was added and incubated for 15 min at 24°C before the second fluorometric reading was performed and blank subtracted. G-6-P concentration was calculated using serially diluted G-6-P stock solutions.

**Measurement of glucose uptake and glycogen synthesis in vivo.** Skeletal muscle glucose uptake and glycogen synthesis in vivo were measured as previously described (12, 29). Briefly, mice were fasted overnight and then anesthetized with pentobarbital sodium. After 30 min, blood was taken from the tail to assess basal glucose and background radioactivity levels. A bolus of 1 mg glucose containing 0.33 \(\mu\)Ci 2-deoxy-\[^3H\]glucose and 0.33 \(\mu\)Ci \[^{14}\text{C}\]glucose/g mouse body wt was administered via a retro-orbital injection, and blood samples were taken, 5, 10, 15, 25, and 35, and 45 min later for the determination of blood glucose, 2-deoxy-\[^3H\]glucose, and \([U-^{14}\text{C}]\)glucose specific activity. After the last blood draw, animals were killed by cervical dislocation, the tibialis anterior muscles from both legs were removed, and muscles were immediately frozen in liquid nitrogen. Accumulation of 2-deoxy-\[^3H\]G-6-P from one muscle was assessed via a precipitation protocol adapted from Ferre et al. (6) using barium hydroxide/zinc sulfate and PCA. The other muscle was processed, and \[^{14}\text{C}\]glucose incorporation into glycogen was measured as described in the previous section.

**RESULTS**

Phosphorylation state and activity of GS in response to insulin and glycogen content in different muscle types. We have previously reported that, although insulin failed to dephosphorylate and activate GS in quadriceps muscle of GSK3\(\alpha/\beta\)S21A/S21A/S9A/S9A mice, the glycogen content in this muscle was similar to that from control wild-type GSK3\(\alpha/\beta\)S21A/S21A/S9A/S9A mice, and insulin stimulation of GS activity was abolished in GSK3 knockin mice and if glycogen levels in the muscles from GSK3 knockin mice were similar to wild-type mice. Basal GS activity in glycolytic white gastrocnemius and glycolytic and oxidative mixed red gastrocnemius muscles was similar between wild-type and GSK3 knockin mice (Fig. 1, A and C). Insulin injection resulted in a robust \(\sim 10\)-fold increase in PKB phosphorylation at Thr\(^{308}\) and \(\sim 30\%\) decrease in GS phosphorylation at Ser\(^{641/645}\) in red (Fig. 1A) and white (not shown) gastrocnemius muscles, as well as \(\sim 2\)-fold increase in GS activity ratio (\(\pm G-6-P\)) in both muscles (Fig. 1, B and C) of wild-type mice. Consistent with our previous results obtained from quadriceps muscle (15), insulin did not promote dephosphorylation (Fig. 1A) and increase of GS activity ratio in red and white gastrocnemius muscles from GSK3 knockin mice (Fig. 1, B and C). The activity of muscle GS in the presence of G-6-P (total activity) was similar between wild-type and GSK3 knockin mice and was not altered by insulin (data not shown). GS phosphorylation at Ser\(^7\)}
was similar in wild-type and GSK3 knockin mice in the basal state, and insulin did not significantly alter the level of phosphorylation at this site in the two genotypes (Fig. 1A).

There was no difference in glycogen content in oxidative soleus and red and white gastrocnemius muscles of wild-type and GSK3 knockin mice either in the fasted or fed state (Fig. 1, D–F).

**Normal glycogen phosphorylase activity in basal and in response to epinephrine in muscle of GSK3α/β<sup>S21A/S21A/S9A/S9A</sup> mice.** Because the net content of muscle glycogen is regulated simultaneously by both synthesis and breakdown, it is possible that the glycogen degradation pathway is altered to compensate for a lack of GSK3-mediated stimulation of glycogen synthesis in response to insulin. To test if this is the case, we measured the activity of glycogen phosphorylase, a rate-limiting enzyme in the pathway of glycogen degradation in muscle. Basal phosphorylase activity ratio (± AMP) and total protein expression in mixed gastrocnemius muscle were similar between wild-type and GSK3 knockin mice (Fig. 2A). We also found that basal phosphorylase activity ratio was similar between wild-type and GSK3 knockin mice in red and white gastrocnemius, as well as soleus muscles (data not shown). Intraperitoneal injection of epinephrine robustly stimulated phosphorylase activity ratio and phosphorylation at Ser<sup>15</sup> by ~2.5- and ~2-fold, respectively (Fig. 2A). As reported previously (16, 28), epinephrine also stimulated GS phosphorylation at Ser<sup>7</sup> by approximately twofold and deactivated GS by ~50% in wild-type mice, and similar results were obtained in GSK3 knockin animals (Fig. 2B). Consistent with the previous report, GS phosphorylation at Ser<sup>641/645</sup> was also modestly increased with epinephrine (28) in both genotypes (data not shown). We also measured glycogen phosphorylase activity and phosphorylation with insulin injection (40 min) and observed no changes in activity or phosphorylation at Ser<sup>15</sup> under this condition in either genotype (Fig. 2C). The phosphorylase activity in the presence of AMP (total activity) was similar between wild-type and GSK3 knockin mice and was not altered by epinephrine or insulin (data not shown).

**Glycogen synthesis and glycogen accumulation in response to insulin in isolated muscle in vitro.** We next determined the rate of glycogen synthesis in response to insulin in isolated soleus muscle incubated in vitro. We first incubated muscles with 5.5 mM glucose containing [14C]glucose in the presence or absence of insulin. In the basal state, in the absence of insulin, the rate of glycogen synthesis in soleus muscle between wild-type and GSK3 knockin mice was similar, and insulin stimulated [14C]glucose incorporation into glycogen to the same extent (~4- to 5-fold increase) in muscles from both genotypes (Fig. 3A). We next measured glycogen content in soleus muscle following incubation for 1 or 2 h with 5.5 mM cold glucose with or without insulin. We observed that insulin significantly increased glycogen content following 1 h incubation (~70–80%) and further increased (~100%) after 2 h of incubation in both wild-type and GSK3 knockin mice (Fig. 3B).

We also measured G-6-P levels in soleus muscle following incubation with 5.5 mM cold (nonradioactive) glucose with or without insulin for 40 min. There was no difference in the level of G-6-P in basal (without insulin), and insulin increased G-6-P content ~60% in muscles from both genotype (Fig. 3C). The level of hexokinase II expression in soleus was similar between wild-type and GSK3 knockin mice (data not shown).

**Glucose uptake and glucose incorporation into glycogen in vivo.** We have measured muscle glucose uptake and glycogen synthesis in vivo by retroorbital injection of glucose containing 2-deoxy-[3H]glucose and [14C]glucose. We observed comparable 2-deoxyglucose uptake and glucose incor-
DISCUSSION

In the present study, we demonstrated that GSK3/S21A/S21A/S9A/S9A/S9A knockin mice displayed a normal capacity to stimulate muscle glycogen synthesis in response to insulin in different muscle types. Our data show that there is no detectable compensatory alteration in the glycogen breakdown pathway, such as a reduced level and activity of glycogen phosphorylase in muscles of GSK3 knockin mice. Moreover, intramuscular G-6-P levels, which allosterically regulate GS activity, were also similar in basal and insulin-stimulated muscles from wild-type and GSK3 knockin mice. Taken together, these results suggest that insulin-mediated inhibition of GSK3 is not a rate-limiting step in muscle glycogen synthesis in mice and that allosteric regulation of GS by G-6-P may play a key role in insulin-stimulated muscle glycogen synthesis in vivo (Fig. 5).

A critical role for muscle GS in glycogen synthesis has been demonstrated by two genetic studies. Manchester et al. (14) demonstrated that overexpression of the constitutively active form of muscle GS (Ser to Ala mutations at sites 2 and 3a) was sufficient to increase muscle glycogen levels up to 10-fold. Pederson et al. (17) generated mice deficient in muscle GS and reported that these animals do not contain detectable levels of muscle glycogen.

It has been well known that GS is regulated by covalent phosphorylation and by the allosteric effector G-6-P (22), but the relative role of these two events in the regulation of GS...
activity in vivo is largely unknown. The major reason for this is because, to our knowledge, there is currently no experimental or assay system to assess G-6-P-mediated regulation of GS in vivo directly. For example, we and others routinely assay GS activity in vitro using muscle extracts in which the rate of incorporation of [14C]UDP-glucose into glycogen is measured in the absence or presence of G-6-P (27). The GS activity in the presence of saturating concentration of G-6-P is independent of the state of phosphorylation, and the activity ratio in the absence relative to that in the presence of G-6-P is used as an index of GS activity. However, it should be noted that the binding of G-6-P to GS is reversible so that the allosteric effect of endogenous G-6-P on GS is lost when muscle tissue is homogenized in a protein extraction buffer and further diluted when GS activity in vitro is assayed.

There is another major reason why it is difficult to assess the relative importance of phosphorylation vs. allosteric activation of GS in the synthesis of glycogen in vivo. Glycogen synthesis requires both glucose uptake and GS activation because glucose, after its conversion to UDP-glucose, serves as substrate for GS to elongate glycogen in addition to acting as an allosteric activator when it is transformed to G-6-P. These events are tightly coupled, and insulin stimulates both glucose transport and GS activation through a phosphoinositide 3-kinase (PI 3-kinase)-PKB-dependent mechanism (24, 25). For this reason, pharmacological inhibitors or genetic mouse models to block PI 3-kinase and/or PKB pathways cannot be used, since they simultaneously inhibit glucose uptake and GS activity. Selective cell-permeable GSK3 inhibitors have been developed, and GS activity was enhanced by these compound (3); however, it should be used with caution, since inhibition of GSK3 may enhance the insulin-signaling pathway through the regulation of insulin receptor substrate-1 (5). The GSK3 knockin mouse model overcomes these inherent problems, since it does not alter insulin-simulated glucose uptake (15) but disables GSK3 to promote dephosphorylation and activation of GS in vivo.

Whether glucose transport into muscle determines the rate of glycogen synthesis in muscle is controversial (13). In skeletal muscle from transgenic mice that overexpress GLUT1, both glucose uptake and the level of glycogen were increased severalfold (20). In contrast, transgenic mice overexpressing GLUT4 resulted in an enhanced rate of glucose transport by ~50%, but these mice possessed similar levels of GS activity and glycogen synthesis rate in muscle compared with control wild-type mice (9). Furthermore, transgenic mice overexpressing the constitutively active form of GS displayed normal rates of glucose uptake but had a pronounced increase in glycogen content up to 10-fold compared with control mice (1). However, it should be noted that the overexpression and/or knock-out approach often creates compensatory/artifactual responses, and results from these studies need to be interpreted carefully. For example, muscles from GLUT4-deficient mice display significantly higher glycogen compared with that of wild-type animals, even though they lack insulin-stimulated muscle glucose uptake. This effect subsequently turned out to be because of multiple compensatory changes in glycogen metabolism (e.g., decreased phosphorylase activity) in muscle of GLUT4-null mice (11).

Although our study suggests that allosteric regulation of GS may play a key role in the synthesis of muscle glycogen in response to insulin, whether this regulatory mechanism plays an essential role for insulin-stimulated glycogen synthesis cannot be addressed from the results of this study. Basal GS activity is still preserved in the muscle of GSK3 knockin animals, and this may be sufficient to maintain normal levels of...
glycogen synthesis. It would be interesting if there is a genetic model in which the binding site of G-6-P is mutated so that GS cannot be activated by G-6-P but maintains its ability to be activated by GSK3. However, to our knowledge, there is no such mutation reported in humans. The complex between GS and G-6-P has not been reported, and the structure of GS does not provide information about the mechanism by which G-6-P interacts with GS and regulates its activity. Studies suggest that, on GS, two clusters of conserved Arg residues (Arg586/588/591) comprise a G-6-P binding site (8, 18). When these Arg sites are all mutated to Ala and expressed in Cos7 cells, they are G-6-P resistant but remain active; however, protein expression of these mutants was slightly lower than wild-type enzyme (8). It would be interesting to express and characterize these mutants in skeletal muscle cells and, if appropriate, generate a knockin mouse model.

In summary, we have demonstrated that, in GSK3α/βS21A/S21A/ S21A/S9A/S9A mice, insulin failed to promote the dephosphorylation and activation of GS in multiple muscle types but possessed a normal capacity to stimulate glycogen synthesis with insulin. We suggest that insulin stimulation of glucose transport and its conversion to G-6-P allosterically activates GS in vivo and plays a vital role in maintaining glycogen levels in muscle. Insulin resistance is often associated with impairment in the signaling pathways that regulate both glucose uptake and GS. Understanding the allosteric regulation of GS in vivo will be important to design new drugs that target GS for the treatment of type 2 diabetes.

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