

## Effects of Insulin and Transgenic Overexpression of UDP-glucose Pyrophosphorylase on UDP-glucose and Glycogen Accumulation in Skeletal Muscle Fibers\*

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**UDP-glucose (UDP-Glc) and glycogen levels in skeletal muscle fibers of defined fiber type were measured using microanalytical methods. Infusing rats with insulin increased glycogen in both Type I and Type II fibers. Insulin was without effect on UDP-Glc in Type I fibers but decreased UDP-Glc by 35–40% in Type IIA/D and Type IIB fibers. The reduction in UDP-Glc suggested that UDP-Glc pyrophosphorylase (PPL) activity might limit glycogen synthesis in response to insulin. To explore this possibility, we generated mice overexpressing a UDP-Glc PPL transgene in skeletal muscle. The transgene increased both UDP-Glc PPL activity and levels of UDP-Glc in skeletal muscles by ~3-fold. However, overexpression of UDP-Glc PPL was without effect on either the levels of skeletal muscle glycogen or glucose tolerance *in vivo*. The transgene was also without effect on either control or insulin-stimulated rates of <sup>14</sup>C-glucose incorporation into glycogen in muscles incubated *in vitro*. The results indicate that UDP-Glc PPL activity is not limiting for glycogen synthesis.**

Glycogen synthesis by skeletal muscle is of critical importance in the control of blood glucose levels by insulin. Skeletal muscle is the major site of insulin-stimulated glucose uptake, and most of the glucose taken up by muscle in response to insulin is deposited as glycogen (1). This synthetic pathway begins with glucose transport, which is activated strongly by insulin (2). Intracellular glucose is phosphorylated rapidly by hexokinase to form glucose 6-phosphate (Glu-6-P),<sup>1</sup> which is isomerized to glucose 1-phosphate (Glu-1-P) by phosphoglucomutase. Uridine diphosphoglucose (UDP-Glc) pyrophosphorylase (PPL) then utilizes UTP and Glu-1-P to form UDP-Glc, the immediate precursor for glycogen synthesis. The final step in which the glucosyl unit from UDP-Glc is used to extend the glycogen polymer is catalyzed by glycogen syn-

thase, which is also activated by insulin (3). Thus, the first and last steps in the glycogen synthetic pathway are controlled by insulin.

Insulin promotes the movement of the glucose transporter, GLUT4, from intracellular compartments to the cell surface (2). The resulting increase in glucose transport and phosphorylation results in an increase in the intracellular concentration of Glu-6-P, which is an allosteric activator of glycogen synthase (3). Glycogen synthase activity is also controlled by multisite phosphorylation (3). Phosphorylation of the appropriate sites inactivates glycogen synthase; however, at sufficiently high concentrations, Glu-6-P is able to activate even highly phosphorylated forms of the enzyme. Insulin promotes dephosphorylation of several sites in glycogen synthase, resulting in an increase in Glu-6-P-independent activity as well as a decrease in the  $K_a$  for Glu-6-P (3). Thus, glycogen synthase activity is coupled to glucose transport via changes in intracellular Glu-6-P (4).

The relative contributions of glucose transport and glycogen synthase to the stimulation of glycogen synthesis by insulin have been debated for many years (3, 4). Studies in transgenic mice have demonstrated that sufficiently increasing either glycogen synthase or glucose transporter proteins in skeletal muscle results in glycogen accumulation (5–8). Elegant noninvasive methods involving NMR spectroscopy have been used to investigate the control of glycogen synthesis *in vivo* (4, 9). Application of these methods has revealed that insulin promotes an increase in Glu-6-P content in skeletal muscle (10), consistent with the stimulatory effect of insulin on glucose transport and phosphorylation. By mass action, an increase in Glu-6-P would be expected to increase levels of downstream intermediates in the glycogen synthesis pathway. However, this is not necessarily the case because a sufficient increase in Glu-6-P would activate glycogen synthase, thereby increasing the rate of utilization of UDP-Glc. A limitation of the NMR method is the lack of sufficient sensitivity to measure such intermediates, and there is evidence that Glu-6-P is not a predictor of levels of UDP-Glc. In glucose-clamped rats, hyperinsulinemia increased Glu-6-P in rectus abdominis muscle, but decreased UDP-Glc (11). Although indicative of significant stimulatory effects of insulin on both glucose transport and glycogen synthase activity, these findings suggest that the UDP-Glc PPL reaction might limit glycogen synthesis in the presence of insulin.

A complicating factor in interpreting the measurements of metabolites *in vivo* is the fact that the fibers found in most skeletal muscles are heterogeneous, differing not only in con-

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<sup>1</sup> The abbreviations used are: Glu-6-P, glucose 6-phosphate; CAT, chloramphenicol acetyltransferase; EDL, extensor digitorum longus; Glu-1-P, glucose 1-phosphate; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PPL, pyrophosphorylase.

tractile speed but also in metabolic potential (12–14). Fibers may be assigned to different classes, or types, based on the activities of representative enzymes of energy metabolism (12–14). Type I fibers have relatively low levels of glycolytic enzymes and high levels of oxidative enzymes. Type I fibers also have a slow twitch speed and are resistant to fatigue because most of their energy is derived from oxidative metabolism. Type IIB fibers have relatively high levels of glycolytic enzymes and low levels of oxidative enzymes. Type IIB fibers also have a fast twitch speed and depend largely on glycogen metabolism to provide the energy to fuel rapid and forceful contractions. Type IIA and IID fibers have a fast twitch speed and high levels of both glycolytic and oxidative enzymes (15). The effect of insulin on glucose transport is highest in muscles composed predominantly of oxidative fiber types (16), and the amount of GLUT4 correlates directly with the levels of enzymes of oxidative energy metabolism, such as malate dehydrogenase (MDH) (17).

The present study was conducted to investigate the role of UDP-Glc PPL in the stimulation of glycogen synthesis by insulin. Using microanalytical techniques to measure directly UDP-Glc and glycogen in single manually dissected muscle fibers of defined muscle fiber type, we demonstrate that insulin decreases UDP-Glc in Type II fibers. We also generated transgenic mice overexpressing UDP-Glc PPL in skeletal muscle to test the hypothesis that UDP-Glc PPL activity limits the insulin-stimulated rate of glycogen synthesis.

#### EXPERIMENTAL PROCEDURES

**Glucose Clamps**—Male rats (Sprague-Dawley, ~250 g) were allowed free access to food and water. Rats were anesthetized with pentobarbital before glucose clamps were applied essentially as described previously (18). Briefly, insulin (10 milliunits/min/kg) or an equal volume of saline was infused via a cannula in the jugular vein. The insulin infusion increased circulating insulin to ~400 microunits/ml. Glucose was monitored continuously in blood obtained from a cannula in the carotid artery, and blood glucose was held constant by adjusting the rate of infusion of a solution containing 30% glucose into the femoral vein. After 2 h, the abdomen was opened and the rectus abdominis muscle was freeze-clamped with aluminum tongs that had been chilled in liquid nitrogen. The muscles were stored in liquid nitrogen then freeze-dried at -35 °C and kept under vacuum at -70 °C before use.

**Single Muscle Fiber Analyses**—Single fibers were dissected manually from the muscle samples (19), and pieces of the fibers for analyses were weighed using a quartz fiber fish pole balance (20). Lactate dehydrogenase (LDH) and MDH activities were measured in pieces of individual fibers as described by Hintz *et al.* (12). Levels of glycogen and UDP-Glc were measured as described by Henry and Lowry (21).

**Generation of Transgenic Mice Overexpressing UDP-Glc PPL**—A DNA fragment containing -3300 to +7 of the mouse creatine kinase gene followed by a BstEII site, a chloramphenicol acetyltransferase (CAT) reporter, an SV40 intron, and a polyadenylation site was excised from p3300MCKCAT (22) with HindIII. After blunting the ends, the fragment was inserted into pSL1180 (Amersham Biosciences) between the StuI site and a blunted KpnI site to generate pSL1180<sup>3300MCKCAT</sup>. A cDNA fragment encoding the larger human skeletal muscle UDP-Glc PPL (form II) was excised using ClaI and SmaI from pH379 (23), which was kindly provided by Dr. H. Y. Chang. The fragment was then inserted between blunted ends of pSL1180<sup>3300MCKCAT</sup> which had been cut with BstEII. Proper orientation of the insert was confirmed by nucleotide sequencing. A fragment containing the 3.3-kb creatine kinase gene sequence, UDP-Glc PPL cDNA, and SV40 sequences was excised with ClaI and EcoRV. The fragment was purified and injected into the pronuclei of fertilized mouse eggs ((C57BL6 × CBA)F1 × (C57BL6 × CBA)F1). Embryos were implanted into pseudopregnant females (Swiss Webster), and transgenic pups were identified by using the PCR to detect CAT sequences in tail DNA. Two founder lines, PPL-1 and PPL-2, were propagated by mating transgene-positive animals to wild type C57BL6 mice.

**Preparation of Muscle Samples for Analyses**—Transgenic mice and wild type littermates were fed *ad libitum*, then anesthetized by subcutaneous injection (1 ml/kg) of a mixture of 40 mg/ml ketamine, 10 mg/ml xylazine, and 1.5 mg/ml acepromazine. Muscles were removed and

either incubated *in vitro* to measure [ $^{14}$ C]glucose into glycogen (described later) or rinsed quickly with chilled (0 °C) phosphate-buffered saline (135 mM NaCl and 10 mM sodium phosphate, pH 7.4) and frozen in liquid nitrogen. The muscles were powdered by using a porcelain mortar and pestle chilled in liquid nitrogen. Samples were stored at -80 °C before extracts were prepared as described below. Extract protein was measured using the method of Smith *et al.* (24).

**Measurements of UDP-Glc and Glycogen in Mouse Muscle**—Muscle powders were weighed and then homogenized (50 mg of tissue/ml of acid) at 0 °C in either 0.3 M perchloric acid (for glycogen measurements) or 0.02 M HCl (for UDP-Glc measurements). The homogenates were centrifuged at  $8,900 \times g$  for 10 min at 4 °C before supernatants were collected for analyses. Glycogen was measured by the amyloglucosidase method described by Passonneau and Lauderdale (25). UDP-Glc was measured by a modification of the fluorometric assay described by Passonneau and Lowry (20). Briefly, the HCl extracts were neutralized with NaOH, then after adding 0.1 mM EDTA (100  $\mu$ l/ml extract), the extracts were boiled for 5 min and centrifuged at  $8,900 \times g$  for 20 min at 4 °C. Aliquots of the supernatants (500  $\mu$ l) were added to 500- $\mu$ l solutions containing 2 mM MgCl<sub>2</sub>, 2 mM NAD<sup>+</sup>, 0.016 unit/ml UDP-Glc dehydrogenase, and 50 mM Tris-HCl, pH 8.1. The samples were incubated for 2 h at 24 °C before the NADH generated was measured fluorometrically. After subtracting blank values from incubations conducted without UDP-Glc dehydrogenase, the UDP-Glc content of the samples was determined from a standard curve generated with known concentrations of UDP-Glc.

**Measurements of UDP-Glc PPL Activity in Mouse Skeletal Muscle**—UDP-Glc PPL activity was measured by modifying the method described by Roach *et al.* (26) for measuring the activity of the enzyme in liver extracts. Powdered muscle was homogenized (100 mg of tissue/ml of buffer) on ice using a glass homogenization tube (Thomas) and a Teflon pestle driven at 1,000 rpm. The homogenization buffer contained 50 mM NaCl, 10 mM NaF, 0.25% Tween 20, 10% glycerol, 0.1 mM dithiothreitol, 500 mM microcystine-LR, 50 mM Tris-HCl, pH 7.4, supplemented with 0.1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin-A. The homogenates were rotated at 4 °C for 45 min, then centrifuged at  $8,900 \times g$  for 30 min at 4 °C. The protein concentration of the extracts were determined (24) and then adjusted to a concentration of 0.2 mg/ml by adding homogenization buffer. Samples (2.5  $\mu$ g of protein, 12.5  $\mu$ l) were added to 800  $\mu$ l of 2 mM MgCl<sub>2</sub>, 0.2 mM UTP, 2  $\mu$ M microcystine-LR, 100 nM okadaic acid, and 50 mM Tris-HCl, pH 8.1. The UDP-Glc PPL reactions were initiated by adding 100  $\mu$ l of 5 mM Glu-1-P. After incubating samples for 20 min at 37 °C, the reactions were terminated by adding 150  $\mu$ l of 100 mM EDTA and boiling for 2.5 min. The samples were then centrifuged at  $8,900 \times g$  for 20 min at 4 °C. An aliquot of supernatant (500  $\mu$ l) was added to 500  $\mu$ l of solution containing 2 mM MgCl<sub>2</sub>, 2 mM NAD<sup>+</sup>, 0.016 unit/ml UDP-Glc dehydrogenase, and 50 mM Tris-HCl, pH 8.1. The samples were incubated at 24 °C for 90 min before the absorbance at 340 nm was measured. The amounts of UDP-Glc formed were determined after subtracting background absorbances determined in incubations conducted without UDP-Glc dehydrogenase.

**[ $^{14}$ C]Glucose into Glycogen**—Incubations were conducted essentially as described previously (5). Briefly, extensor digitorum longus (EDL) and diaphragm muscles were removed from anesthetized mice. The diaphragm was dissected into two hemidiaphragms, leaving a rib attached to each to avoid cutting fibers. Muscles were incubated at 37 °C for 30 min to remove endogenous hormones, then transferred to tubes containing 10 ml of Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM potassium phosphate, 1.2 mM MgSO<sub>4</sub>, and 25 mM NaHCO<sub>3</sub>, pH 7.4) plus 5 mM [ $^{14}$ C]glucose (~500 cpm/nmol). The buffer was gassed directly by bubbling with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The muscles were incubated at 37 °C without or with porcine insulin for 30 min, then rinsed quickly, blotted on tissue paper, and frozen in liquid nitrogen.

Muscles were homogenized in 0.4 ml of 30% KOH, before samples were placed in a boiling water bath for 1 h. After adding 50  $\mu$ l of a 5% glycogen solution to each sample, ethanol was added to a final concentration of 70%, and glycogen was allowed to precipitate at -20 °C for 8 h. The samples were centrifuged at  $8,900 \times g$  for 20 min at 4 °C to pellet the glycogen. The glycogen pellets were washed four times with 66% ethanol and dissolved in 50  $\mu$ l of deionized water. Samples were spotted on 2-cm squares of filter paper (Whatman 31ET), which were washed five times with 66% ethanol. The papers were dried before the amount of  $^{14}$ C-labeled glycogen was determined by scintillation counting.

**Intravenous and Intraperitoneal Glucose Tolerance Tests**—Food was removed the evening before glucose tolerance tests, which were conducted the following morning. Mice were injected either intraperitone-

ally with 1.0 mg of glucose/g of body weight or intravenously via the lateral tail vein with 0.5 mg of glucose/g of body weight. The glucose solutions were prepared in 0.9% saline and warmed to 37 °C prior to injections. Blood glucose levels were determined in samples of tail vein blood (~3 µl) at increasing times after the glucose injection using a glucometer (ONE TOUCH FastTake, LifeScan).

**Preparation of Antibodies and Detection of UDP-Glc PPL by Immunoblotting**—A synthetic peptide (CELSVKKELEKILT) having a sequence identical to positions 29–41 of the human UDP-Glc PPL enzyme (23) was coupled to keyhole limpet hemocyanin by using maleimidobenzoyl-*N*-hydroxysuccinimide, and the conjugate was used to immunize rabbits as described previously (27). Antibodies were purified using a column containing an affinity resin prepared by coupling the peptide to Sulfolink beads (Pierce). For immunoblotting, muscle extract samples (25 µg) were subjected to SDS-PAGE in 7.5% polyacrylamide gels (28) before proteins were transferred electrophoretically to Immobilon membranes (Millipore). After incubating with 2 µg/ml UDP-Glc PPL antibodies, the membranes were washed, and antibody binding was detected using alkaline phosphatase-conjugated secondary antibody and Tropix reagent.

**Other Materials**—[U-<sup>14</sup>C]Glucose was obtained from PerkinElmer Life Sciences. Porcine insulin and UDP-Glc dehydrogenase were obtained from Calbiochem. Most other chemicals and reagents were from Sigma.

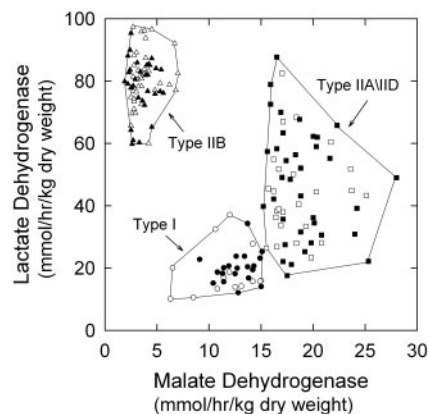
## RESULTS

**Effect of Insulin on Glycogen and UDP-Glc in Single Muscle Fibers**—To investigate the effect of insulin on glycogen synthesis in different types of muscle fibers, rats were infused with either a maximally effective concentration of insulin or a saline control. Blood glucose was clamped at 5 mM to prevent hypoglycemia in the insulin-treated animals and to eliminate differences in blood glucose in the control and insulin-treated animals. Rectus abdominis muscles were freeze-clamped *in situ* then freeze-dried at -30 °C. Muscle fibers were dissected from the dried muscles, and enzyme activities and levels of UDP-Glc and glycogen were measured in pieces of individual fibers.

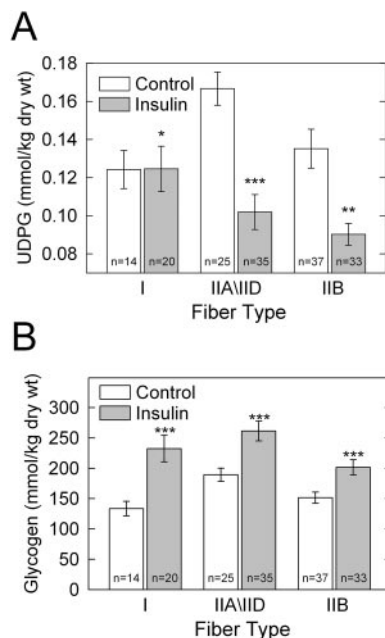
To allow assignment of fibers into different fiber type categories, the activities of LDH and MDH activities were measured (12). Type IIB fibers have the lowest levels of MDH activity of the different types of fibers, and in a plot of LDH versus MDH activity IIB fibers were tightly clustered and well separated from the more oxidative fibers (Fig. 1). Of the oxidative fiber types, Type I fibers have the lowest levels of MDH, and a previous study demonstrated that those fibers with low LDH activity with MDH activity less than 15 mmol/h/kg were Type I (29). In the original study, those fibers having MDH activities greater than 15 mmol/h/kg were categorized as Type IIA fibers (12). It is now known that IID fibers may also have high levels of oxidative enzymes (30). Therefore, fibers having an MDH activity higher than 15 mmol/h/kg were assigned to a IIA/IID category. By these criteria, 20.7% of the fibers were Type I, 36.6% were IIA/D, and 42.7% were IIB.

In muscles from control (saline-infused) animals, the glycogen contents of the three types of muscle fibers ranged from an average of 140 mmol/kg, dry weight, in Type I fibers to 194 mmol/kg, dry weight, in Type IIA/D fibers. Insulin increased the amount of glycogen in all three types of fibers (Fig. 2A). The percentage change caused by insulin was greatest in Type I fibers, where glycogen was increased by 71%. Type IIB fibers exhibited the smallest percentage increase in glycogen levels in response to insulin. Insulin was without effect on the level of UDP-Glc in Type I fibers; however, insulin decreased the UDP-Glc content of both Type IIA/D fibers and Type IIB fibers by 40% ( $p < 0.001$ ) and 28% ( $p < 0.01$ ), respectively (Fig. 2B). The findings in these fast twitch fiber types are consistent with the previous report of decreased UDP-Glc in whole rectus abdominis muscles from insulin-treated rats (11).

**Transgenic Overexpression of UDP-Glc PPL in Skeletal Muscle**—The finding that UDP-Glc concentrations were decreased



**FIG. 1. Fiber type assignments of rectus abdominis fibers from control and insulin-treated rats.** Rats were infused with saline (open symbols) or insulin (10 milliunits/min/kg) (filled symbols), while clamping blood glucose at 5 mM. This required infusing glucose at  $2.9 \pm 0.75$  and  $142 \pm 4$  µmol/min/kg in the control and insulin-treated animals, respectively. After 2 h, the rectus abdominis muscle was freeze-clamped *in situ*. Hind limb glucose uptake measured over this time was increased from  $0.20 \pm 0.4$  to  $0.52 \pm 0.13$  µmol/min by insulin. Single fibers were dissected from three control and three insulin-stimulated muscles. LDH and MDH activities were measured in individual fibers. Based on these activities the fibers were divided into the following three categories: Type I, Type IIA/D, and Type IIB fibers, identified as described by Hintz *et al.* (12). This strategy does not distinguish between IIA and IID fibers, so these fibers are grouped together. The mean ( $\pm$  S.E.) LDH activities (in mmol/hr/kg, dry weight) in the control and insulin-treatment groups, respectively, were as follows: Type I,  $18.7 \pm 2.2$  and  $20.2 \pm 1.1$ ; Type IIA/D,  $44.1 \pm 2.9$  and  $47.2 \pm 3.0$ ; Type IIB,  $81.6 \pm 1.6$  and  $80.0 \pm 1.8$ . MDH activities were as follows: Type I,  $12.0 \pm 0.9$  and  $12.8 \pm 0.4$ ; Type IIA/D,  $18.4 \pm 0.5$  and  $19.0 \pm 0.5$ ; and Type IIB,  $3.7 \pm 0.2$  and  $3.5 \pm 0.2$ .



**FIG. 2. Effects of insulin on levels of UDP-Glc and glycogen in single fibers.** Levels of glycogen in equivalents of glucose (A) and UDP-Glc (B) were measured in single muscle fibers, which were divided into the fiber type categories described in the legend to Fig. 1. Mean values  $\pm$  S.E. (where  $n$  = number of fibers) are presented. The significance of differences between insulin and control values were evaluated by a two-tailed heteroscedastic *t* test (36). \*, not significantly different; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

by insulin suggests that the supply of UDP-Glc by the UDP-Glc PPL reaction limits glycogen accumulation in response to insulin. If this hypothesis were correct, then overexpressing UDP-Glc PPL would be expected to enhance insulin-stimulated



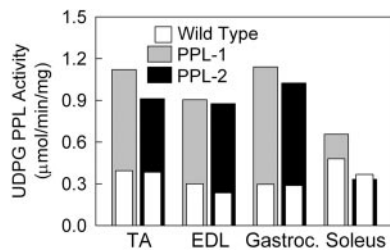


FIG. 3. **Transgenic overexpression of UDP-Glc PPL.** Tibialis anterior (TA), EDL, gastrocnemius (Gastroc.), and soleus muscles were dissected from two lines of mice, PPL-1 and PPL-2, expressing the UDP-Glc PPL transgene and from wild type littermates. UDP-Glc PPL activity was measured in extracts of these muscles and expressed relative to extract protein.

glycogen synthesis. To test this hypothesis, transgenic mice were generated in which UDP-Glc PPL was selectively overexpressed in skeletal muscle by using promoter and enhancer elements from the skeletal muscle creatine kinase gene (22). Transgenes under the control of these elements have been shown to be expressed preferentially in glycolytic muscle fiber types (22). Two transgenic lines, designated PPL-1 and PPL-2, were selected for initial experiments.

UDP-Glc PPL activities in tibialis anterior, EDL, gastrocnemius, and soleus muscles from transgenic animals and wild type littermates were measured (Fig. 3). The four wild type muscles contained comparable amounts of UDP-Glc PPL activity. Activities were 2–3-fold higher in transgenic tibialis anterior, EDL, and gastrocnemius muscles than in these muscles in nontransgenic littermates; however, very little, if any, increase in UDP-Glc PPL activity attributable to the transgene was noted in the soleus muscles (Fig. 3). The soleus is composed predominantly of Type I fibers (31). Thus, expression of UDP-Glc PPL is consistent with the expression of the creatine kinase gene, which is lowest in Type I muscle fibers (22). Because UDP-Glc PPL activities were approximately the same in muscles from PPL-1 and PPL-2 mice, subsequent studies were conducted with only one of the lines (PPL-1).

To confirm transgenic overexpression, antibodies to UDP-Glc PPL were generated, and the levels of enzyme protein were assessed by immunoblotting. The results in Fig. 4 depict immunoblots of samples of quadriceps, EDL, and diaphragm muscles from PPL-1 mice and nontransgenic littermates. The transgene increased UDP-Glc PPL protein by ~5-fold in the quadriceps and EDL muscles. As expected, transgenic expression of UDP-Glc PPL was relatively low in the diaphragm, which is composed primarily of oxidative fibers. Although the percentage increase in enzyme protein in the EDL was somewhat greater than the percentage increase in enzyme activity, the immunoblotting and enzyme activity measurements confirm that UDP-Glc PPL was overexpressed in muscles from PPL-1 mice.

Transgenic overexpression of UDP-Glc PPL increased levels of UDP-Glc in gastrocnemius muscles by ~3-fold (Fig. 5). The percentage increase in UDP-Glc was very similar to the increase in UDP-Glc PPL activity produced by the transgene in these muscles, solidifying the conclusion that the overexpressed enzyme was active. In contrast, levels of glycogen were almost identical in gastrocnemius muscles from PPL-1 mice and nontransgenic littermates (Fig. 5). Thus, increasing UDP-Glc, the immediate precursor of glycogen, by transgenic overexpression of UDP-Glc PPL was not sufficient to drive glycogen accumulation.

To investigate the effect of increasing UDP-Glc on insulin action *in vivo*, we evaluated the effect of the transgene on glucose tolerance by conducting both intraperitoneal (Fig. 6A) and intravenous (Fig. 6B) glucose tolerance tests. The changes in blood glucose after administration of glucose in nontrans-

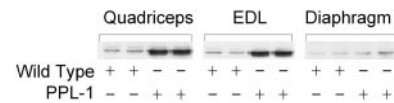


FIG. 4. **Relative levels of UDP-Glc PPL achieved by transgenic overexpression in different skeletal muscles.** Quadriceps, EDL, and diaphragm muscles were dissected from two PPL-1 mice and from two nontransgenic littermates. Samples of extracts were subjected to SDS-PAGE and immunoblotted with antibodies to UDP-Glc PPL.

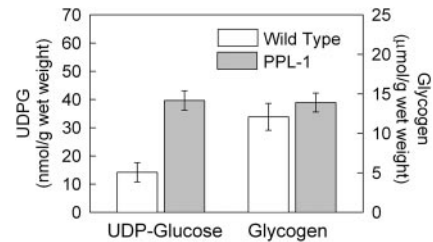


FIG. 5. **Effects of transgenic overexpression of UDP-Glc PPL on the UDP-Glc and glycogen contents in skeletal muscle.** Glycogen and UDP-Glc were measured in samples of quadriceps muscles from PPL-1 mice and nontransgenic littermates. The results are expressed relative to wet weight of the tissues and are the mean values  $\pm$  S.E. of measurements obtained from muscles of three wild type mice and four transgenic mice.

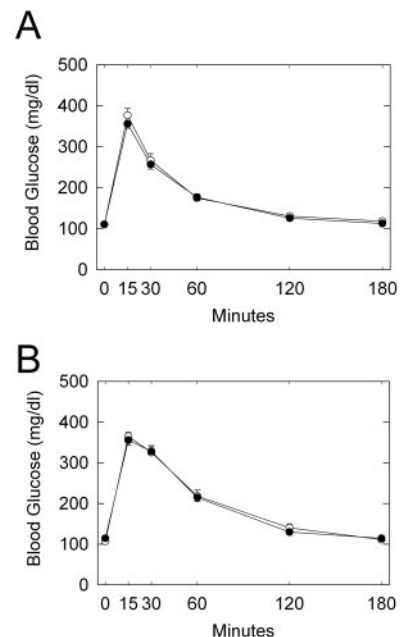


FIG. 6. **Glucose tolerance is unaffected by transgenic overexpression of UDP-Glc PPL.** PPL-1 mice (●) and nontransgenic littermates (○) were fasted overnight. A, blood glucose was measured at increasing times after injecting glucose (1 mg/g of body weight) intraperitoneally. Mean values  $\pm$  S.E. from 27 wild type mice and 33 transgenic mice are presented. B, blood glucose was measured at increasing times after injecting glucose (0.5 mg/g of body weight) intravenously. Mean values  $\pm$  S.E. from 28 wild type and 34 transgenic mice are presented.

genic and transgenic animals were virtually identical. Thus, transgenic overexpression of UDP-Glc PPL in skeletal muscle did not significantly affect whole animal glucose disposal.

**Failure of Transgenic Overexpression of UDP-Glc PPL to Enhance Insulin-stimulated Glycogen Synthesis *In Vitro***—As a result of homeostatic mechanisms, a change in insulin action in skeletal muscle is not always apparent in glucose tolerance tests. To examine more directly insulin action in skeletal muscle, we conducted *in vitro* experiments with wild type and transgenic EDL muscles. After incubating in medium contain-

TABLE I

Effect of insulin on UDP-Glc and Glu-6-P levels in wild type and transgenic EDL muscles incubated *in vitro*

EDL muscles from PPL-1 mice and nontransgenic mice were incubated for 30 min at 37 °C without or with 250 milliunits/ml insulin in Krebs-Ringer bicarbonate buffer containing 5 mM glucose before levels of UDP-Glc and Glu-6-P were measured as described previously (7). The results presented are mean values  $\pm$  S.E. from three wild type and five transgenic muscles.

Treatment	UDP-Glc in EDL from		Glu-6-P in EDL from	
	Wild type	PPL-1	Wild type	PPL-1
	nmol/g, wet weight		nmol/g, wet weight	
Control	7.8 $\pm$ 0.8	11.8 $\pm$ 1.6*	58 $\pm$ 6	59 $\pm$ 6
Insulin, 250 milliunits/ml	4.3 $\pm$ 0.4**	6.4 $\pm$ 0.2***	82 $\pm$ 20	77 $\pm$ 7

\*  $p < 0.05$ , significance of difference between PPL-1 and wild type.  
\*\*  $p < 0.01$ , significance of difference between insulin and control.

ing 5 mM glucose, levels of UDP-Glc in the EDL muscles (Table I) were lower than those measured in quadriceps that had been freeze-clamped *in situ* (Fig. 5). Thus, incubation *in vitro* may decrease UDP-Glc levels, although it is also possible that UDP-Glc levels are different in quadriceps and EDL muscles. Importantly, incubating the EDL muscles with insulin decreased UDP-Glc levels by  $\sim 45\%$  (Table I). This change, which is comparable with the percentage change produced by insulin in Type II muscle fibers *in vivo* (Fig. 2B), confirms that insulin decreases UDP-Glc in mouse skeletal muscle.

Levels of UDP-Glc were 50% higher in EDL muscles from PPL-1 mice than in muscles from wild type animals (Table I), although the effect of the transgene was not as large as in quadriceps frozen *in situ* (Fig. 5). In contrast, levels of Glu-6-P in wild type and transgenic muscles were not significantly different, in either the absence or presence of insulin (Table I). Transgenic overexpression of PPL-1 did not prevent the fall in UDP-Glc in response to insulin. However, after incubation with insulin, UDP-Glc was 50% higher in transgenic muscles than in wild type muscles.

Experiments were conducted to determine whether the increase in UDP-Glc produced by the transgene was sufficient to increase glycogen synthesis. Incubating wild type EDL muscles *in vitro* with a physiological concentration of insulin (100 microunits/ml) increased [U-<sup>14</sup>C]glucose incorporation into glycogen by  $\sim 2$ -fold (Fig. 7A). With a maximally effective concentration of insulin, the amount of [U-<sup>14</sup>C]glucose incorporated into glycogen was increased by 15-fold (Fig. 7B). The transgene did not significantly affect [<sup>14</sup>C]glycogen synthesis in either the absence or presence of insulin (Fig. 7, A and B). Results comparable with those in the EDL muscles were obtained with nontransgenic and transgenic hemidiaphragms (Fig. 7C).

DISCUSSION

The present results indicate that insulin decreases UDP-Glc levels in Type II skeletal muscle fibers (Fig. 2A). This finding suggested to us that UDP-Glc PPL activity might limit glycogen synthesis in fast twitch muscle fibers stimulated with insulin. To test this hypothesis, we generated transgenic animals overexpressing UDP-Glc PPL in skeletal muscle. The results demonstrate that the transgene increased UDP-Glc in muscle (Fig. 5) but was without effect on glycogen accumulation (Fig. 5) and glucose tolerance *in vivo* (Fig. 6) or on rates of glucose incorporation into glycogen *in vitro* (Fig. 7).

The striking metabolic heterogeneity of the fibers that compose skeletal muscle is a complicating factor that is rarely addressed in interpreting the meaning of changes in metabolites measured in whole muscles or in animals. When the effects of insulin on metabolite levels in skeletal muscle are

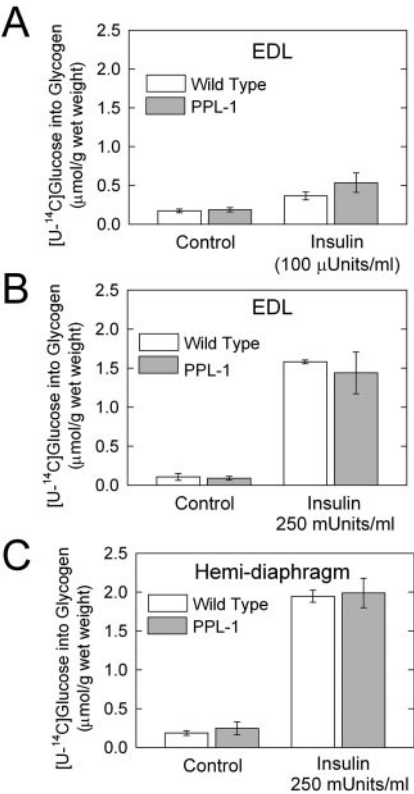


FIG. 7. Failure of UDP-Glc PPL overexpression to increase [U-<sup>14</sup>C]glucose incorporation into glycogen *in vitro*. Muscles from PPL-1 mice and nontransgenic littermates were incubated for 30 min at 37 °C without or with either 100 microunits/ml insulin (A) or 250 milliunits/ml insulin (B and C) in Krebs-Ringer bicarbonate buffer containing 5 mM [U-<sup>14</sup>C]glucose (500 cpm/nmol). [U-<sup>14</sup>C]Glucose incorporation into glycogen was determined and expressed relative to the wet weight of the tissues. Mean values  $\pm$  S.E. from eight wild type and eight transgenic EDL muscles (A), four wild type and four transgenic EDL muscles (B), and three wild type and three transgenic hemidiaphragms (C) are presented.

investigated, it is common practice to assume that the changes occur within the same muscle fibers, although this is not necessarily the case. We addressed this potential problem in a unique manner using microanalytical methods to measure levels of UDP-Glc and glycogen in single manually dissected muscle fibers from control and insulin-treated animals. Insulin increased glycogen and decreased UDP-Glc levels in Type II fibers, whereas in Type I fibers the hormone increased glycogen but did not decrease UDP-Glc (Fig. 2).

Our results confirm and extend the observations of Rossetti and Hu (11), who found that infusing rats with insulin decreased UDP-Glc levels in whole rectus abdominis muscles. In principle, a decrease in UDP-Glc could result from either an increase in its rate of utilization or a decrease in its rate of formation. Because insulin stimulates glucose transport/phosphorylation, it is unlikely that a decrease in the supply of Glu-1-P provided by isomerization of Glu-6-P explains the fall in UDP-Glc. Glu-1-P is also produced by the phosphorylase reaction, and there is evidence that insulin decreases glycogenolysis in skeletal muscle (32, 33). If the rate of glycogenolysis were sufficiently high, inhibiting the process could lead to a decrease in UDP-Glc. However, as suggested previously (11), it seems more likely that the well established effect of insulin on activating glycogen synthase, which utilizes UDP-Glc to synthesize glycogen, explains the fall in UDP-Glc. Consistent with this interpretation, transgenic overexpression of glycogen synthase decreased UDP-Glc in skeletal muscle (7). In the present study insulin decreased UDP-Glc in both control and PPL-1

EDL muscles (Table I). Indeed, the absolute decrease in UDP-Glc produced by insulin was larger in the transgenic muscles than in the wild type muscles (5.4 nmol/g, wet weight, compared with 3.5 nmol/g, wet weight), indicating that insulin activated glycogen synthase in the transgenic muscles.

The intracellular concentration of UDP-Glc appears to be insufficient to saturate glycogen synthase.<sup>2</sup> Therefore, regardless of the mechanism through which insulin decreases UDP-Glc, such a decrease would be expected to limit the rate of glycogen synthesis. Likewise, all things being equal an increase in UDP-Glc should increase glycogen synthesis. For this reason the finding that increasing UDP-Glc PPL in skeletal muscle did not lead to glycogen accumulation was unexpected. It is clear that the transgene was expressed. Immunoblotting with antibodies to UDP-Glc PPL demonstrated that enzyme protein was increased in different skeletal muscles in a pattern consistent with that expected from the creatine kinase promoter and enhancer elements that were used to drive transgenic expression (Fig. 4). Measurements of UDP-Glc PPL activity indicated that the amount of the transgene expressed was sufficient to increase UDP-Glc PPL activity (Fig. 3). Moreover, measurements of tissue UDP-Glc confirmed that the amount of UDP-Glc PPL overexpressed was sufficient to increase UDP-Glc in skeletal muscle (Fig. 5).

The finding that UDP-Glc levels can be increased without affecting glycogen synthesis argues that UDP-Glc PPL does not limit glycogen synthesis; however, there are caveats attached to this conclusion. We cannot be certain that the transgene increased UDP-Glc in the same pool as that utilized for glycogen synthesis. Moreover, as with other studies involving transgenic animals, long term overexpression of an enzyme may produce unknown compensatory changes that complicate interpretation of the results. The present findings are similar in some respects to those of Skurat *et al.* (34), who found that overexpressing UDP-Glc PPL in COS cells, either alone or with wild type glycogen synthase, was without effect on glycogen accumulation. Although additional studies will be needed to determine why increasing UDP-Glc by transgenic overexpression of UDP-Glc PPL does not increase glycogen accumulation,

the present findings are indicative of the complex mechanisms involved in the control of glycogen metabolism by insulin.

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<sup>2</sup> Measurements of the  $K_m$  of purified rabbit muscle glycogen synthase for UDP-Glc ranged from approximately 50  $\mu$ M for the fully dephosphorylated form assayed in the presence of Glu-6-P to more than 9 mM for a phosphorylated enzyme assayed in the absence of Glu-6-P (35). The dry weight of single muscle fibers is approximately 20% of the wet weight. To estimate the concentration of UDP-Glc from the single fiber measurements, it was assumed that 1 kg, wet weight, was equal to a 1-liter volume and that UDP-Glc was distributed evenly within the cell. Thus, for example, after insulin treatment the UDP-Glc concentration in Type II fibers was approximately 50  $\mu$ M.

**Effects of Insulin and Transgenic Overexpression of UDP-glucose  
Pyrophosphorylase on UDP-glucose and Glycogen Accumulation in Skeletal Muscle  
Fibers**

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