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Increased glycogen accumulation in transgenic mice overexpressing glycogen synthase in skeletal muscle

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ABSTRACT **To investigate the role of glycogen synthase in controlling glycogen accumulation, we generated three lines of transgenic mice in which the enzyme was overexpressed in skeletal muscle by using promoter-enhancer elements derived from the mouse muscle creatine kinase gene. In all three lines, expression was highest in muscles composed primarily of fast-twitch fibers, such as the gastrocnemius and anterior tibialis. In these muscles, glycogen synthase activity was increased by as much as 10-fold, with concomitant increases (up to 5-fold) in the glycogen content. The uridine diphosphoglucose concentrations were markedly decreased, consistent with the increase in glycogen synthase activity. Levels of glycogen phosphorylase in these muscles increased (up to 3-fold), whereas the amount of the insulin-sensitive glucose transporter 4 either remained unchanged or decreased. The observation that increasing glycogen synthase enhances glycogen accumulation supports the conclusion that the activation of glycogen synthase, as well as glucose transport, contributes to the accumulation of glycogen in response to insulin in skeletal muscle.**

Diabetes mellitus is a common disease, which occurs in approximately 3% of the general population of the United States (1). Complications resulting from diabetes are among the leading causes of heart disease, renal insufficiency, and blindness (1). The hallmark of the untreated disease is elevated blood glucose. It is now clear that maintaining blood glucose at near normal levels may prevent or delay the appearance of diabetic complications (2). Insulin has a pivotal role in lowering blood glucose by stimulating glucose uptake and storage in various insulin-sensitive tissues. Therefore, it is critical to understand the mechanisms involved in insulin stimulation of glucose uptake.

Skeletal muscle accounts for the bulk of postprandial glucose uptake (3–6) and most of the glucose that enters muscle fibers in response to insulin is converted to glycogen (7, 8). The synthesis of glycogen occurs via a pathway that begins with the transport of glucose across the plasma membrane. Intracellular glucose is phosphorylated by hexokinase to glucose-6-phosphate (G6P), which is then sequentially converted to glucose-1-phosphate (G1P) and uridine diphosphoglucose (UDPG) in reactions catalyzed by phosphoglucomutase and UDPG pyrophosphorylase, respectively. In the final step in the pathway, the glucosyl moiety from UDPG is added to preexisting glycogen in a reaction catalyzed by glycogen synthase. Although it is well established that insulin activates both glucose transport and glycogen synthase in skeletal muscle, a controversy has arisen regarding the role of glycogen synthase activation in insulin-stimulated glycogen synthesis (9, 10).

Glycogen synthase is subject to complex control by multisite phosphorylation and by several allosteric effectors, of which the activator G6P is most important (11, 12). In general, phosphorylation decreases synthase activity, but the extent of inactivation depends on the sites phosphorylated and on the effector concentrations. Even though phosphorylation may increase the concentration of G6P required for activation, G6P fully activates even highly phosphorylated forms of the enzyme. Two phosphorylation sites (2 and 2a) are present in the first 10 amino acids at the NH_2 terminus of skeletal muscle glycogen synthase, whereas at least seven (3a, 3b, 3c, 4, 5, 1a, and 1b) are found in a stretch of 100 amino acids at the COOH terminus (11, 12). In recent studies in COS cells, the influence of different phosphorylation sites on the activity ratio (−G6P/+G6P) was investigated by expressing glycogen synthases in which sites were mutated to Ala. Mutations of site 2, together with either site 3a or 3b, were required to significantly increase the activity ratio (13, 14) and to increase the accumulation of glycogen (14, 15). In skeletal muscle, insulin promotes dephosphorylation of site 2 and site 3 (a + b + c), decreases the K_a for G6P, and increases the activity ratio (16).

Insulin-stimulated glucose transport in skeletal muscle occurs by facilitative diffusion mediated by the transport protein, GLUT4 (17, 18). Under basal conditions, very little GLUT4 is found at the cell surface. Insulin promotes translocation of GLUT4 from intracellular compartments to the plasma membrane, thereby increasing glucose transport. Skeletal muscle fibers also express GLUT1, another member of the glucose transporter family (19). Because the levels of GLUT1 are normally much lower than those of GLUT4, GLUT1 is not believed to contribute significantly to the insulin-stimulated component of glucose transport in skeletal muscle (17, 18). However, GLUT1 may contribute to basal glucose uptake, since a large fraction of this transporter is found at the cell surface, even in the absence of insulin (19). Overexpressing GLUT1 in muscle cells markedly increases glucose transport even in the absence of insulin (10, 20). Skeletal muscles from transgenic mice overexpressing GLUT1 exhibited a severalfold increase in glycogen, but the glycogen synthase activities measured in extracts were not significantly different from those in muscles from nontransgenic littermates (10). Based on these results, it has been concluded that glucose transport is the rate-limiting step in the synthesis of glycogen in skeletal muscle (10, 21). More recently it has been argued that the activation of glycogen synthase does not serve to increase the flux of glucose into glycogen, but rather to control levels of intermediates in the pathway, such as G6P (9). While this model would be consistent with the results of the GLUT1 transgenic studies, it is difficult to understand why the complex regulation of synthase would have evolved if the enzyme was not also a controlling factor in glycogen deposition.

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Abbreviations: GLUT1 and GLUT4, two members of the facilitative diffusion family of glucose transporters; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; UDPG, uridine diphosphoglucose.

To investigate further the role of glycogen synthase in the control of glycogen synthesis *in vivo*, we used a transgenic approach to increase glycogen synthase activity in mouse skeletal muscle. The results show that increasing glycogen synthase markedly increases glycogen accumulation *in vivo*, supporting the conclusion that regulation of glycogen synthase is important for controlling glycogen synthesis.

MATERIALS AND METHODS

Generation of Transgenic Mouse Lines. Increasing glycogen in skeletal muscle is associated with inactivation of glycogen synthase (22). Therefore, to limit the inactivation of synthase, we chose to express GS(2,3a), a rabbit skeletal muscle glycogen synthase having Ser to Ala mutations in both site 2 and site 3a (14). cDNA encoding GS(2,3a) was inserted into the *Bst*EII site of p3300MCKCAT (23). Just upstream of this *Bst*EII site is the sequence from −3300 to +7 of the mouse creatine kinase gene, which includes elements that direct expression to both skeletal and cardiac muscle (23, 24). However, transgene expression is typically greater than 30 times higher in skeletal muscles with predominantly fast-twitch fiber types than in cardiac muscle; and, expression in leg muscles that are composed of fast-twitch fibers, such as the extensor digitorum longus, is generally about 5 times higher than in those composed of slow-twitch fibers, such as the soleus (25). Immediately adjacent on the downstream side of the *Bst*EII site is a chloramphenicol acetyltransferase reporter followed by a simian virus 40 intron and polyadenylylation site. After digesting p3300MCKCAT with *Bst*EII, the vector was blunt-ended and dephosphorylated. A fragment containing GS(2,3a) cDNA was excised from pCMV-GS(2,3a) (14) by using *Bgl*II and *Xba*I, then blunt-ligated into p3300MCKCAT. Proper orientation of the insert was confirmed by nucleotide sequencing. A fragment containing the 3.3-kb creatine kinase gene sequence, GS(2,3a) cDNA, and simian virus 40 sequences was excised using *Hin*dIII followed by a partial digest with *Kpn*I, inserted between the *Hin*dIII and *Kpn*I sites of pSL1180 (Pharmacia), then excised with *Hin*dIII and *Eco*RV. The 7.5-kb fragment was purified and injected into the pronuclei of fertilized mouse eggs [(C57BL6 × CBA)F_1 × (C57BL6 × CBA)F_1] (26). Embryos were implanted into pseudopregnant females (Swiss–Webster) and transgenic pups were identified by using the polymerase chain reaction to detect chloramphenicol acetyltransferase sequences in tail DNA. Three founder lines (GSL3, GSL25, and GSL30) were established by mating transgene-positive animals to (C57BL6 × CBA)F_1 mice.

Preparation of Tissue Extracts. Four-month-old transgenic and control animals were fed *ad libitum*, then anesthetized by subcutaneous injection (1 ml/kg) of a mixture of ketamine (40 mg/ml), xylazine (10 mg/ml), and acepromazine (1.5 mg/ml). After cervical dislocation, individual muscles and other tissues were dissected and frozen in liquid nitrogen. Tissues were homogenized in buffer (30 μl per mg tissue wet weight) containing 0.5 mM dithiothreitol, 50 mM NaF, 5 mM EDTA, and 50 mM Tris·HCl (pH 7.8). The homogenates were centrifuged at 10,000 × *g* for 5 min, and the supernatants were removed. The protein concentration was determined by the method of Bradford (27). To obtain tissue for freeze-clamp analyses, hindlimb muscles were exposed and immediately frozen *in situ* between two stainless steel blocks (1 cm × 2.5 cm × 2 cm) that had been chilled in liquid nitrogen. When metabolite levels were measured, muscle was freeze-dried at −30° before extracts were prepared by homogenizing samples in 0.02 N HCl (10 μl per mg tissue dry weight).

Measurements of Enzymes and Metabolites. Glycogen synthase activity was determined by measuring the incorporation of [U-^{14}C]glucose from UDP[U-^{14}C]glucose into glycogen (28). Total glycogen synthase activity is defined as that determined in the presence of 10 mM G6P (28). The activity ratio (−G6P/+G6P) was determined by dividing the activity measured without added G6P by the total activity. Total phosphorylase activity was measured in the direction of glycogen synthesis from [U-^{14}C]G1P by using a reaction mixture containing 3 mM 5′ AMP (29). Extracts of freeze-clamped muscles were neutralized with NaOH before G6P, G1P, UDPG, and glycogen were measured using the methods described by Lowry and Passonneau (30) for determining the concentrations of these metabolites in tissue extracts.

Electrophoretic Analyses. Samples of extracts or homogenates were mixed with buffer containing SDS and subjected to SDS/PAGE by using the method of Laemmli (31). After transfer to nylon membranes (Immobilon, Millipore), GLUT4 (20) and glycogen synthase (32) were detected by immunoblotting. Relative amounts of the proteins were determined by two-dimensional scanning of films by using a laser densitometer (Molecular Dynamics).

RESULTS

Detection of Transgenic Glycogen Synthase. To assess the amount of glycogen synthase expressed from the transgene, extracts from gastrocnemius muscles were subjected to SDS/PAGE and an immunoblot was prepared (Fig. 1). All three transgenic lines exhibited increased levels of glycogen synthase protein, with the level of overexpression relative to control ranging from approximately 2-fold in GSL25 to more than 10-fold in GSL30. Glycogen synthase protein from all samples appeared as several electrophoretically distinguishable species (Fig. 1), most likely because of differences in phosphorylation that is known to retard the electrophoretic mobility of the glycogen synthase subunit (33). Transgenic glycogen synthase had a slightly higher electrophoretic mobility than the endogenous mouse enzyme. Although the difference in mobility could represent an inherent difference in molecular mass between the transgenic enzyme (derived from rabbit) and the mouse enzyme, we believe the higher relative mobility of the transgenic enzyme is due to a lower phosphate content, as is seen in the GS(2,3a) mutant when expressed in COS cells (14).

Expressing the mutant glycogen synthase not only increased total synthase activity (Fig. 2) but also increased the activity ratio. Activity ratios (−G6P/+G6P) measured in extracts from gastrocnemius muscles that had been freeze-clamped *in situ* were as follows: Control, 0.121 ± 0.017; GSL25, 0.12 ± 0.005; GSL3, 0.179 ± 0.008; and GSL30 0.193 ± 0.024. Activity ratios in transgenic animals were lower than observed in expression studies in COS cells, where expressing GS(2,3a) results in ratios of >0.5 (14). Factors determining the activation state of glycogen synthase in skeletal muscle may differ from those prevailing in COS cells. Also, glycogen synthase is multimeric and a hybrid of phosphorylated and dephosphorylated subunits appears to be inactive (34). Thus, the level of expression of the mutant relative to the endogenous enzyme is

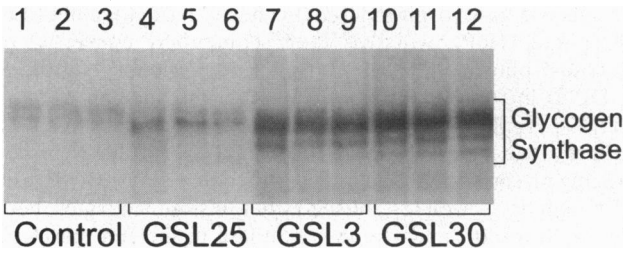

FIG. 1. Overexpression of glycogen synthase in transgenic animals. Samples (25 μg protein) of freeze-clamped gastrocnemius muscles from three control animals and each transgenic line were dissolved in SDS sample buffer and subjected to SDS/PAGE. After transfer to nylon membranes, glycogen synthase was detected by enhanced chemiluminescence using glycogen synthase antibodies (32).

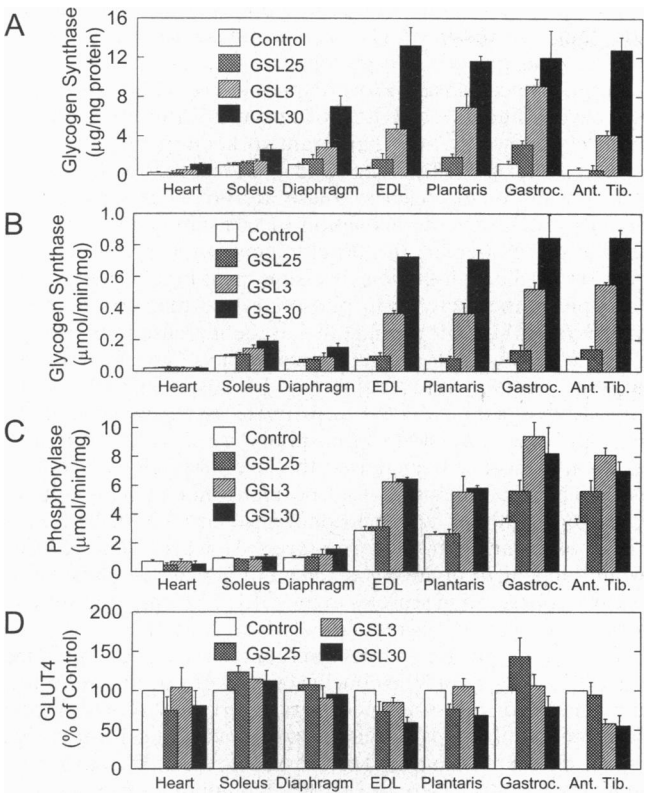

FIG. 2. Levels of glycogen synthase, phosphorylase, and GLUT4 in different muscles. Samples of heart and various skeletal muscles were homogenized, and extracts were prepared. The amounts of glycogen synthase protein (*A*) in extracts were determined by immunoblotting, using two-dimensional laser densitometry to estimate band intensities. Purified rabbit skeletal muscle glycogen synthase was used as a standard to estimate the amount of glycogen synthase (micrograms) per mg of extract protein. Total activities of glycogen synthase (*B*) and phosphorylase (*C*) were measured in extracts. Relative levels of GLUT4 (*D*) in homogenates were estimated by immunoblotting, and are expressed as percentages of the GLUT4 found in the respective muscles from control animals. The results are mean values ± SEM from muscles obtained from three animals.

important in determining the activity ratio. In the COS cell experiments (14), the degree of overexpression was greater than in the transgenic animals.

Pattern of Glycogen Synthase Overexpression. The creatine kinase gene sequence used for expressing the transgene contains enhancer elements directing muscle specific expression (23, 24, 35). As expected, no increase in glycogen synthase was observed in brain, liver, kidney, or spleen (results not shown), and expression in heart was characteristically low relative to that in skeletal muscle (Fig. 2*A*). The level of overexpression in skeletal muscle correlated with the muscle fiber composition. The soleus, which contains a relatively high proportion of slow-twitch muscle fibers (36), expressed relatively little of the transgene. Muscles composed primarily of fast-twitch fibers, such as the extensor digitorum longus, plantaris, gastrocnemius, and anterior tibialis (36), expressed relatively high levels of glycogen synthase. These differences in expression of transgenic glycogen synthase are consistent with the lower expression of endogenous muscle creatine kinase in slow-twitch muscle fibers (37, 38), as well as with expression of other transgenic proteins driven by muscle creatine kinase regulatory gene cassettes (24, 35).

In all muscles, the order of overexpression among the transgenic lines, GSL30 > GSL3 > GSL25, was preserved. In general, the amount of total glycogen synthase activity (Fig. 2*B*) in extracts agreed well with the amount of glycogen synthase estimated by immunoblotting (Fig. 2*A*). Exceptions were noted in the heart and in the diaphragm, where less activity was observed than would have been predicted from the amount of enzyme protein detected. The reason for this discrepancy is not clear.

GLUT4 levels in heart, soleus, and diaphragm were not significantly different in transgenic and control animals. However, the GLUT4 contents of extensor digitorum longus, plantaris, gastrocnemius, and anterior tibialis muscles in GSL30, the transgenic line expressing the highest levels of glycogen synthase, were significantly lower than those in the corresponding muscles of control animals (Fig. 2*D*). Interestingly, in transgenic muscles exhibiting relatively large increases in glycogen synthase, the levels of phosphorylase were increased by as much as 2-fold (Fig. 2*C*).

Accumulation of Glycogen in Muscles from Transgenic Mice. To determine whether increasing synthase affected glycogen synthesis, glycogen was measured in samples from freeze-clamped gastrocnemius and anterior tibialis muscles (Fig. 3). Transgenic muscles contained up to five times more glycogen than the control. The glycogen level was generally proportional to the amount of glycogen synthase expressed.

To understand better the metabolic changes resulting in the accumulation of glycogen, each metabolite in the pathway leading to glycogen synthesis was measured (Fig. 4). In the gastrocnemius muscles the level of G6P increased significantly in GSL30, but it was not changed in GSL3 where glycogen was also markedly increased. In anterior tibialis muscles G6P was also slightly elevated in GSL30, but was 40% lower in GSL3 than in the control. G1P levels were not significantly different from control in any of the transgenic muscles. The levels of UDPG were much lower in muscles from all three transgenic lines, except the anterior tibialis from GSL25.

DISCUSSION

The finding that transgenic expression of an activated form of glycogen synthase *in vivo* resulted in a marked increase in skeletal muscle glycogen indicates that glycogen synthase activity is important in controlling the levels of tissue glycogen. Another implication is that the activation of glycogen synthase is important in the stimulation of glycogen accumulation by insulin.

We stress that glycogen synthase activity need not be the only factor controlling glycogen synthesis. Studies of trans-

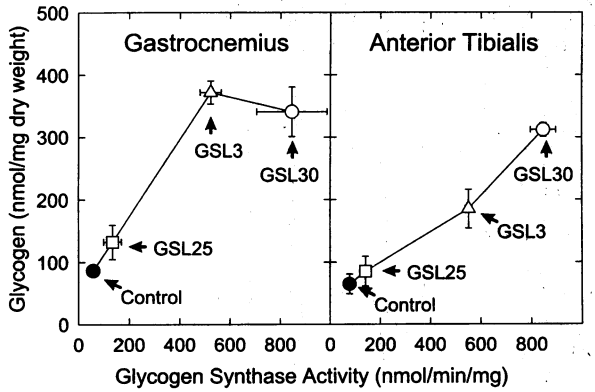

FIG. 3. Correlation between levels of glycogen synthase and glycogen accumulation. Mice were anesthetized before hindlimb muscles were exposed and freeze-clamped *in situ*. Levels of glycogen and total glycogen synthase activities were measured in extracts of gastrocnemius and anterior tibialis muscles. Glycogen synthase activities are expressed relative to extract protein. Values for glycogen represent the glucose equivalents in glycogen and are expressed relative to the dry weight of the tissue. The results represent means ± SEM of muscles from three animals. Error bars not shown fall within the symbol.

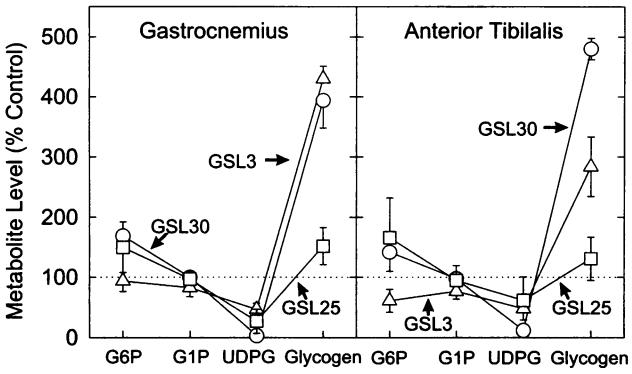

FIG. 4. Crossover analysis of the glycogen synthesis pathway. Metabolites in the pathway leading to the synthesis of glycogen from intracellular glucose were measured in extracts from freeze-clamped gastrocnemius and anterior tibialis muscles. The results from the transgenic lines represent means ± SEM from three animals and are expressed as percentages of the values measured in muscles from control animals. The levels of metabolites in the control gastrocnemius muscles (expressed as nmol per mg dry weight) were as follows: G6P, 0.71 ± 0.11; G1P, 0.072 ± 0.003; UDPG, 0.048 ± 0.010; and glycogen (glucose equivalents), 108 ± 8.2. Levels in the control anterior tibialis muscles were as follows: G6P, 0.56 ± 0.19; G1P, 0.06 ± 0.009; UDPG, 0.023 ± 0.005; and glycogen, 65 ± 16.

genic animals in which glucose transport was increased by overexpressing GLUT1 in muscle (10) demonstrated that increased glucose entry leads to net glycogen accumulation comparable to that observed by overexpressing glycogen synthase (Fig. 3). However, if glucose transport were the only determining step in glycogen synthesis, as has been suggested previously (10), then increasing glycogen synthase activity independently of glucose transport should not increase glycogen accumulation.

In the glucose transporter/hexokinase (GT/HK) model recently proposed by Shulman and coworkers (9), the activation of glycogen synthase by insulin would not control the rate of glycogen synthesis, but rather serve to adapt the activity of the enzyme to the metabolic flux and to prevent overaccumulation of metabolites. The present results support a more direct role for glycogen synthase activation in the control of glycogen synthesis, because increasing glycogen synthase enhanced glycogen accumulation (Fig. 3). Being downstream of GLUT4 in the metabolic pathway, glycogen synthase is dependent on glucose transport to provide substrate for glycogen synthesis. The comprehensive *in vivo* glucose-clamp analyses of Rossetti and Hu (39) have demonstrated that the activation of glycogen synthase *in vivo* occurs at lower concentrations of insulin than the stimulation of glucose uptake, results that also seem inconsistent with the GT/HK model. At insulin concentrations that did not increase glucose transport or accumulation of glycogen, glycogen synthase activation was found to prevent net glycogenolysis by stimulating the reincorporation of glucose equivalents into glycogen (39). Thus, glycogen synthase activation by insulin is likely to be important even when transport is not stimulated.

UDPG concentrations were decreased in our transgenic animals as a result of the increased synthase activity (Fig. 4), and the lack of substrate probably limited glycogen accumulation. Increasing insulin has also been shown to decrease the levels of both UDPG and G6P prior to activation of glucose transport (39). The decrease in UDPG is indicative of an action of insulin-activated glycogen synthase to "pull" intracellular glucose into glycogen. Earlier experiments with preparations of cut muscle fibers, which glucose may enter by simple diffusion, support this concept (40). When glucose uptakes in the absence and presence of insulin were matched by varying the concentrations of glucose in the medium, glycogen synthesis was higher in insulin-treated preparations (40), indicating that activation of glycogen synthase served to direct intracellular glucose into glycogen.

In principle, transgenic overexpression provides a means to selectively alter the activities of enzymes within a metabolic pathway. However, it is important to keep in mind that the effects observed with long-term overexpression of glucose transporters or glycogen synthase are not necessarily representative of the acute activation of transport or synthase by insulin. In particular, the development of compensatory responses to the transgenic expression must be considered. An example is the increase in phosphorylase that was noted in GSL3 and GSL30. It is unlikely that the increase in phosphorylase contributed to the accumulation of glycogen in the present experiments, as increased phosphorylase activity would have been expected to promote glycogenolysis. Interestingly, overexpression of phosphorylase in cultured human skeletal muscle cells increased the level of glycogen synthase (41). Thus, it is tempting to speculate that the amounts of glycogen synthase and phosphorylase are controlled in a reciprocal manner by glycogen levels. However, other factors would appear to be involved since no increase in phosphorylase was observed in muscles in the GLUT1 transgenic animal, where glycogen was also markedly increased (10).

Studies of muscles from transgenic mice overexpressing GLUT4 also support the conclusion that glycogen synthesis is not regulated solely by glucose transport activity. Using perfused hindlimb preparations, Brozinick *et al.* (42) found that 4-fold overexpression of skeletal muscle GLUT4 was associated with a 50% increase in the maximum rate of insulin-stimulated glucose transport but no change in the rate of glycogen synthesis. Hansen *et al.* (43) reported that isolated extensor digitorum brevis muscles from GLUT4 transgenic mice exhibited a 2-fold higher basal rate of glucose transport but no difference in the basal rate of glycogen synthesis. More recent findings even provide reason to question the view that GLUT4 is required for glucose homeostasis. Katz *et al.* (44) found that female mice, lacking GLUT as a result of targeted gene disruption, are capable of maintaining near normal levels of blood glucose. Presumably the GLUT4 "knockout" animals compensate by using another transporter (21). However, it will be interesting to determine whether the compensatory response involves intracellular effects of insulin, such as the activation of glycogen synthase.

In summary, it seems reasonable to conclude that under appropriate conditions an increase either in glucose transport or in glycogen synthase activity is sufficient to stimulate the accumulation of glycogen. The relative importance of the two may depend on the physiological or pathological setting.

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