

# Glycogen Synthase Sensitivity to Insulin and Glucose-6-Phosphate Is Mediated by Both NH<sub>2</sub>- and COOH-Terminal Phosphorylation Sites

Alexander V. Skurat, Amy D. Dietrich, and Peter J. Roach

**In skeletal muscle, insulin activates glycogen synthase by reducing phosphorylation at both NH<sub>2</sub>- and COOH-terminal sites of the enzyme and by elevating the levels of glucose-6-phosphate, an allosteric activator of glycogen synthase. To study the mechanism of regulation of glycogen synthase by insulin and glucose-6-phosphate, we generated stable Rat-1 fibroblast clones expressing rabbit muscle glycogen synthase with Ser→Ala substitutions at key phosphorylation sites. We found that 1) elimination of the phosphorylation of either NH<sub>2</sub>- or COOH-terminal sites did not abolish insulin stimulation of glycogen synthase; 2) mutations at both Ser-7 and Ser-640 were necessary to bypass insulin activation; 3) mutation at Ser-7, coupled with the disruption of the motif for recognition by glycogen synthase kinase-3 (GSK-3), did not eliminate the insulin effect; and 4) mutation of either Ser-7 or Ser-640 increased the sensitivity of glycogen synthase to glucose 6-phosphate >10-fold. We conclude that Ser-7 and Ser-640 are both involved in mediating the response of glycogen synthase to insulin and activation by glucose 6-phosphate. In Rat-1 fibroblasts, GSK-3 action is not essential for glycogen synthase activation by insulin, and GSK-3-independent mechanisms also operate. *Diabetes* 49:1096-1100, 2000**

**A** major metabolic consequence of insulin action is an increase in glycogen levels, especially in liver and muscle. A key enzyme in the regulation of glycogen synthesis is glycogen synthase, which is controlled by multisite phosphorylation and the binding of allosteric ligands (1-3). Phosphorylation leads to inactivation of glycogen synthase, but activity is restored by the allosteric activator glucose-6-phosphate. In mammalian glycogen synthase, phosphorylation sites are located at both NH<sub>2</sub>- and COOH-termini (Fig. 1). Of the 7 COOH-terminal phosphorylation sites, Ser-640 (site 3a) and Ser-644 (site 3b) are the most

important for regulation of glycogen synthase (4). At the NH<sub>2</sub>-terminus, both Ser-7 (site 2) and Ser-10 (site 2a) influence glycogen synthase activity (5,6). Insulin administration causes dephosphorylation of glycogen synthase and, consequently, activation of the enzyme. In one study, insulin treatment of 24-h-starved rabbits decreased the total phosphate from 2.7 to 2.3 mol per glycogen synthase polypeptide with the greatest decrease within the region containing phosphorylation sites 3a, 3b, and 3c (7). Even in this study, dephosphorylation of NH<sub>2</sub>-terminal sites was reported, and several other investigations (8-10) have shown that insulin promotes dephosphorylation of sites 2 and/or 2a, as well as site 1b.

Insulin stimulation of glycogen synthase most likely occurs via a signaling pathway involving phosphatidylinositol 3-kinase (PI3-kinase). One current hypothesis is that activation of PI3-kinase by insulin results in phosphorylation and activation of protein kinase B, which in turn phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3) (11). In vitro, GSK-3 phosphorylates sequentially sites 4, 3c, 3b, and 3a in glycogen synthase, but recognition of site 4 by GSK-3 requires that glycogen synthase has first been phosphorylated at site 5 (12-14). The molecular basis appears to be that GSK-3 modifies residues in the sequence motif S-X-X-X-S(P), for which S indicates serine, P indicates phosphate, and X indicates any amino acid (15). However, more recent work has shown that sites 3a and 3b can also be directly phosphorylated by currently unidentified protein kinases (4,16). The role, if any, of these protein kinases in insulin signaling to glycogen synthase has yet to be demonstrated. Insulin also has been reported to activate the glycogen-associated protein phosphatase type 1 (PP1G) by phosphorylation of the regulatory subunit of PP1G (R<sub>GL</sub>) at Ser-48 (17). Despite extensive study, the exact signaling pathways linking the activation of PP1G to the insulin receptor are not well understood, and the role of R<sub>GL</sub> in insulin signaling has been challenged by the observation that R<sub>GL</sub> knockout mice have normal insulin stimulation of glycogen synthase (18).

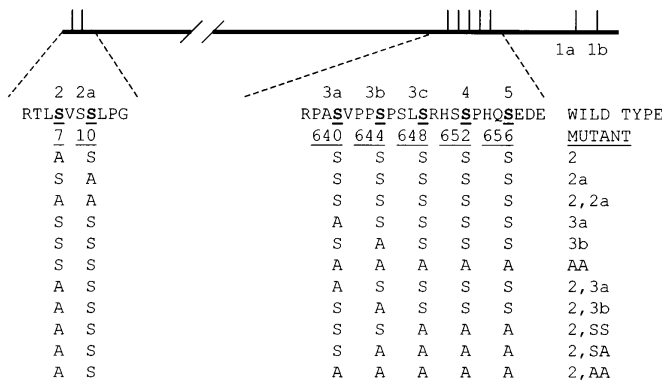
Dephosphorylation of glycogen synthase, such as occurs in insulin action, causes significant changes in enzyme kinetic properties that result generally in increased activity. Thus, activity measured in the absence of glucose-6-phosphate is increased due to an increased affinity for the substrate UDP-glucose. In addition, the concentration of glucose-6-phosphate required for half-maximal stimulation ( $M_{0.5}$ ) is decreased ~1,000-fold for dephosphorylated glycogen synthase compared with species with a high phosphate content (19,20). The sensitivity to glucose-6-phosphate was shown to be determined by phosphorylation of specific regions in

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana.

Address correspondence and reprint requests to Alexander V. Skurat, PhD, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202. E-mail: askurat@iupui.edu.

Received for publication 17 December 1999 and accepted in revised form 29 March 2000.

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GSK-3, glycogen synthase kinase-3; HB, homogenization buffer;  $M_{0.5}$ , half-maximal stimulation; PI3-kinase, phosphatidylinositol 3-kinase; PP1G, glycogen-associated protein phosphatase type 1; R<sub>GL</sub>, regulatory subunit of PP1G.



**FIG. 1. Arrangement of phosphorylation sites in wild-type and mutated glycogen synthase.** The horizontal line represents rabbit muscle glycogen synthase; the vertical tick marks indicate phosphorylation sites. The amino acid sequence of 2 regions containing phosphorylation sites, as underlined, is given. The designation of the phosphorylation sites and residue numbers, also as underlined, are shown. For the mutants, the amino acids in the positions corresponding to each phosphorylation site, as well as their respective names, are indicated.

glycogen synthase. Incorporation of 1.2 mol/subunit of phosphate into rabbit skeletal muscle glycogen synthase catalyzed by GSK-3 caused a 15-fold increase in  $M_{0.5}$  for glucose-6-phosphate. However, phosphorylation to a similar extent with either cyclic AMP-dependent protein kinase or phosphorylase kinase produced smaller changes in activity (21). The contribution of the individual phosphorylation sites to the sensitivity of glycogen synthase to glucose-6-phosphate was not analyzed in these studies.

Previously, we had constructed a series of glycogen synthase mutants with Ser→Ala substitutions at different phosphorylation sites. Expression of the mutants in COS cells followed by measurements of the enzymatic activities and phosphorylation resulted in identification of the critical phosphorylation sites involved in regulation of glycogen synthase (4,5). In the present work, we used some of these same mutations (Fig. 1) for stable expression in Rat-1 fibroblasts as a cell model to study insulin stimulation of glycogen synthase. We show that both sites 2 (and/or 2a) and 3a are involved in mediating the sensitivity of glycogen synthase to insulin and glucose-6-phosphate.

## RESEARCH DESIGN AND METHODS

**Stable expression of glycogen synthase in Rat-1 fibroblasts.** Construction of cytomegalovirus promoter-driven expression vector containing rabbit muscle glycogen synthase cDNA that encodes wild-type or mutated proteins has been described previously (4,5). Each of the expression plasmids was mixed with pCMVneo vector for co-transfection of Rat-1 fibroblasts using LipofectAMINE (Life Technologies, Rockville, MD). Stable clones were obtained by selection in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.8 mg/ml G418 (Life Technologies). The levels of expression of glycogen synthase in G418-resistant clones were determined by the measurement of the specific activity of glycogen synthase. Transfection of Rat-1 fibroblasts with pCMVneo alone resulted in the generation of G418-resistant clones, one of which was used as a control.

**Insulin treatment and preparation of cell extracts.** Stable clones of Rat-1 fibroblasts were cultured to confluent monolayers in 60-mm dishes in DMEM with 25 mmol/l glucose supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.4 mg/ml G418, followed by 48-h incubation in the same media with 5 mmol/l glucose. The cells were incubated in serum- and glucose-free DMEM for 3 h, and porcine insulin (Eli Lilly, Indi-

anapolis, IN) was added to a final concentration 100 nmol/l. After incubation for 1 h, cells were rinsed with 6 ml of ice-cold homogenization buffer (HB) (50 mmol/l Tris-HCl, pH 7.8, 10 mmol/l EDTA, 2 mmol/l EGTA, 100 mmol/l NaF, 0.1 mmol/l  $N^{\alpha}$ -*p*-tosyl-L-lysine chloromethyl ketone, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 mmol/l dithiothreitol) and were frozen in liquid nitrogen in 0.5 ml of HB buffer. After thawing, lysed cells were collected by scraping with a rubber policeman and were homogenized by repeated pipetting. Homogenates were centrifuged at 16,000g for 15 min, and pellets were resuspended in 0.5 ml HB buffer. The supernatants and resuspended pellets were designated as the soluble and pellet fractions, respectively.

**Glycogen synthase assay.** Glycogen synthase activity was determined by measuring the incorporation of [ $^{14}$ C]glucose from UDP-[U- $^{14}$ C]glucose into glycogen (22). For standard activity assays, soluble or pellet fractions (30 µl) were incubated at 30°C with a 60-µl assay mixture containing 50 mmol/l Tris-HCl, pH 7.8, 25 mmol/l potassium fluoride, 20 mmol/l EDTA, 1% glycogen, 6.67 mmol/l UDP-[ $^{14}$ C]glucose (specific activity 200 cpm/nmol). The reaction was carried out in the absence or presence of glucose-6-phosphate at different final concentrations. For the standard -/+ glucose-6-phosphate activity ratio, glucose-6-phosphate was present at 7.2 mmol/l. For the measurement of fractional velocity (23), the low glucose-6-phosphate condition was 30 µmol/l and the high concentration was 7.2 mmol/l. The activity was expressed as the amount of glucose transferred from UDP-glucose to glycogen in 1 min/mg of cell extract protein. The activity of the pellet fraction was expressed in terms of the protein in the corresponding soluble fraction to allow direct comparison between the 2 measurements.

Protein was quantitated by the method of Bradford (24) using bovine  $\gamma$ -globulin as the standard.

## RESULTS

**Generation of Rat-1 cell lines stably expressing glycogen synthase.** In a previous work, we studied the effects of Ser→Ala substitution at individual phosphorylation sites on the activation state of glycogen synthase transiently expressed in COS cells (4,5). Although this cell model was effective for identification of the phosphorylation sites having the greatest effects on glycogen synthase activity, it did not allow us to evaluate their role in insulin signaling. COS cells respond poorly to insulin, and the transfected cell population is heterogeneous, with high levels of expression in only a fraction of the cells. Thus, we chose to make stable clones of Rat-1 fibroblasts overexpressing wild-type and mutated glycogen synthase. Consistent with the subcellular distribution of expressed glycogen synthase observed in COS cells (25), glycogen synthase was found predominantly in the low-speed pellet fractions of Rat-1 cell homogenates. As was shown with COS cell expression, the expressed glycogen synthase was active in the presence of glucose-6-phosphate (Table 1) and was co-sedimented with glycogen, which over-accumulated (data not shown), indicating that the protein is functional inside the cell. The activity state of wild-type protein measured in the absence of glucose-6-phosphate was extremely low; this finding is consistent with hyperphosphorylation of the glycogen synthase and corresponds to a -/+ glucose-6-phosphate activity ratio close to zero. Therefore, for some determinations, the activation states of wild-type and several mutated glycogen synthases were estimated as the ratio of the activity measured in the presence of 30 µmol/l glucose-6-phosphate to that measured in the presence of 6.7 mmol/l glucose-6-phosphate (Table 1). This parameter, sometimes called fractional velocity, has often been used to make activity changes easier to measure in analyses of the effects of insulin and other hormones on glycogen synthase (23). Another group of mutants with Ser→Ala substitutions at both  $NH_2$ - and  $COOH$ -termini was characterized by higher glucose-6-phosphate-independent activity, and, therefore, it was possible to monitor the alterations in their activation state as changes in the standard -/+ glucose-6-phosphate activity ratio.

TABLE 1  
Effect of mutations of phosphorylation sites on the activation state of glycogen synthase expressed in Rat-1 cells

Mutation	Glycogen synthase total activity		Glycogen synthase activation state		<i>n</i>	<i>P</i> *
	Control	Insulin	Control	Insulin		
			Fractional velocity†			
Wild type	62 ± 19	67 ± 17	0.014 ± 0.004	0.032 ± 0.007	4	0.01
2	111 ± 50	103 ± 39	0.069 ± 0.026	0.125 ± 0.035	5	0.001
2a	129 ± 29	106 ± 20	0.092 ± 0.013	0.13 ± 0.026	3	0.039
2,2a	137 ± 33	132 ± 22	0.043 ± 0.01	0.068 ± 0.016	3	0.016
3a	46 ± 10	45 ± 12	0.062 ± 0.007	0.106 ± 0.013	3	0.012
3b	60 ± 10	66 ± 5	0.038 ± 0.003	0.066 ± 0.013	3	0.038
AA	19 ± 4	20 ± 4	0.027 ± 0.012	0.062 ± 0.025	3	0.048
			Activity ratio‡			
2	111 ± 50	103 ± 39	0.016 ± 0.007	0.039 ± 0.015	5	0.002
2,3b	116 ± 19	129 ± 27	0.048 ± 0.008	0.065 ± 0.007	4	0.001
2,SS	66 ± 8	65 ± 11	0.035 ± 0.007	0.05 ± 0.009	4	0.017
2,SA	42 ± 7	39 ± 3	0.123 ± 0.028	0.164 ± 0.046	3	0.059
2,3a	188 ± 70	187 ± 63	0.22 ± 0.022	0.215 ± 0.028	3	ND
2,AA	17 ± 3	19 ± 4	0.42 ± 0.015	0.42 ± 0.03	3	ND

Data are means ± SE of duplicate determinations for the number (*n*) of experiments indicated. \*Significance of difference in fractional velocity and activity ratio between glycogen synthases from control and insulin-treated cells. †Glycogen synthase fractional velocity was estimated as a ratio of specific activity measured in the presence of 30 μmol/l glucose-6-phosphate to the specific activity measured in the presence of 7.2 mmol/l glucose-6-phosphate. Fractional velocity was used to characterize wild-type glycogen synthase and the mutants 2; 2a; 2,2a; 3a; 3b; and AA. ‡Glycogen synthase activity ratio is the specific activity measured without glucose-6-phosphate divided by the specific activity measured in the presence of 7.2 mmol/l glucose-6-phosphate. Activity ratio was used to characterize the mutants 2; 2,3b; 2,SS; 2,SA; 2,3a; and 2,AA. ND, not determined.

Overexpression of wild-type glycogen synthase in the clone analyzed in Table 1 increased the total enzyme activity (+glucose-6-phosphate) >30-fold in the pellet fraction of the Rat-1 cells (activity of endogenous glycogen synthase in the pellets from control clones is ~1.8 nmol · min<sup>-1</sup> · mg<sup>-1</sup>). There was significant variability in the absolute levels of enzyme activity between different clones expressing the same glycogen synthase mutant, but there was no strong correlation between the expression level and either the fractional velocity or the degree of the insulin activation. Therefore, phosphorylation in the cells did not depend on expression level. For further analysis, we selected the clones with the highest glycogen synthase activities. Some mutants (most notably AA and 2,AA) (Fig. 1) were expressed at lower levels than wild-type enzymes in all of the clones analyzed (Table 1). It is possible that some phosphorylation sites are important to the stability of the expressed proteins. In our previous work (5), we found that sites 1a and 1b were not important in determining glycogen synthase activity, and hence we did not include mutations in these sites in the present study. Reference to the COOH-terminal phosphorylation sites signifies sites 3a, 3b, 3c, 4, and 5.

**Insulin activation of glycogen synthase.** Incubation of Rat-1 clones expressing wild-type glycogen synthase with insulin caused a 2.3-fold increase in fractional velocity (Table 1 and Fig. 2). Elimination of the key regulatory sites (2, 2a, 3a, or 3b [mutants 2, 2a, 3a, and 3b]) (Fig. 1) individually did not abolish the ability of insulin to activate glycogen synthase, implying that the insulin response involves dephosphorylation of multiple sites in glycogen synthase. Likewise, mutation either of both NH<sub>2</sub>-terminal sites (mutant 2,2a) (Fig. 1) or of 5 COOH-terminal sites (mutant AA) (Fig. 1) did not eliminate the insulin effect (Fig. 2). Phosphorylation sites 3a, 3b, 3c, and 4, which were missing in the mutant AA, are

targets for GSK-3. Therefore, the results suggest that regulation of GSK-3 activity is not sufficient to account for the insulin effect. These results also suggest that insulin regulates both NH<sub>2</sub>- and COOH-terminal phosphorylation in glycogen synthase. In a previous work, we found that a Ser→Ala substitution at site 2 abolishes phosphorylation of the second NH<sub>2</sub>-terminal regulatory site 2a (5). Therefore, combining a mutation at site 2 with mutations at sites 3, 4, and 5 allowed us to analyze the relative contributions of the individual COOH-terminal phosphorylation sites in insulin signaling.

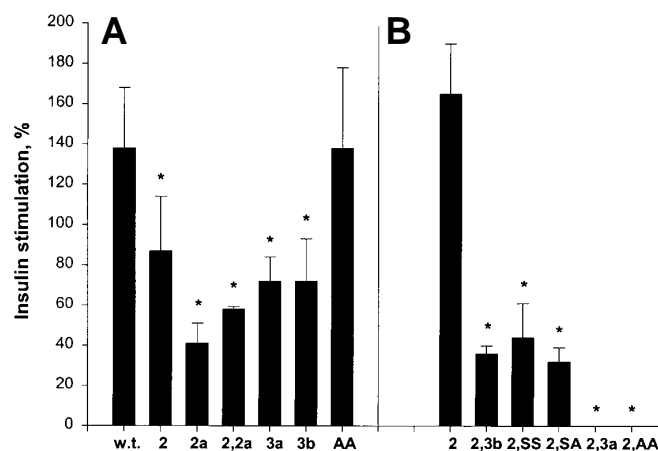


FIG. 2. Stimulation of wild-type and mutated glycogen synthase by insulin. For this figure, the data from Table 1 were used. The bars represent the percentage of change in fractional velocity (A) or activity ratio (B) for wild-type and mutated glycogen synthase as a result of insulin administration. Results are means ± SE of duplicate determinations for the number of experiments indicated in Table 1. \**P* < 0.05 relative to values for wild-type glycogen synthase (A) or mutant 2 (B).

Mutations at sites 2 and 3b (mutant 2,3b) (Fig. 1) or at sites 2, 3c, 4, and 5 (mutant 2,SS) (Fig. 1) did not eliminate insulin stimulation (Fig. 2). An important characteristic of these mutants is the disruption of the consensus motif for GSK-3, S-X-X-S(P) (15). Therefore, although the presence of site 3a or sites 3a and 3b correlated with insulin activation of the enzyme, these results suggest that the regulation in Rat-1 cells does not occur exclusively via inhibition of GSK-3. Site 3a appeared to be important for insulin action. In cells expressing the mutant 2,SA, in which only site 3a remains of the regulatory COOH-terminal sites (see Fig. 1), there was still a trend towards insulin activation ( $P = 0.059$ ) (Table 1). To test whether GSK-3 can phosphorylate site 3a in mutant 2,SA, the mutant was expressed and purified from *Escherichia coli*. No detectable phosphorylation of the recombinant mutant by GSK-3 was observed (A.V.S., Q. Wang, P.J.R., unpublished data). This is consistent also with previous in vitro phosphorylation of recombinant glycogen synthase with mutations only of COOH-terminal sites (26). The importance of site 3a in insulin regulation of glycogen synthase was also supported by the observed inability of insulin to activate glycogen synthase in the cell line expressing the mutant 2,3a (Fig. 2). Although the basal activation state of glycogen synthase in these cells was elevated, it was still 2 times lower than the activity in cells expressing the mutant 2,AA (Table 1), which is not phosphorylated in the regulatory COOH-terminal sites. Thus, there was the potential for further activation of the glycogen synthase by insulin. From these data, we infer that insulin action involved dephosphorylation of site 3a in glycogen synthase and that the mechanism is independent of GSK-3. The lack of GSK-3-dependent insulin-induced activation of glycogen synthase in Rat-1 cells could be explained by the presence of other protein kinases that phosphorylate regulatory sites 3a and 3b. It is possible that, in other cell types, a different balance among the activities of these protein kinases, GSK-3, and protein phosphatases could allow for cell-specific mechanisms for insulin signaling to glycogen synthase.

**Sensitivity to glucose-6-phosphate.** According to the aforementioned studies, insulin activation of wild-type glycogen synthase involves dephosphorylation of both  $\text{NH}_2$ - and COOH-terminal sites, as judged by changes in activity measurements based on the sensitivity to the allosteric activator glucose-6-phosphate. In earlier works, it was shown that phosphorylation decreases the sensitivity of glycogen synthase to glucose-6-phosphate (19,20). The increase in apparent concentration required for half-maximal activation ( $K_a$ ) for glucose-6-phosphate was found to be greater after phosphorylation of glycogen synthase by GSK-3, as compared with the effect of cAMP-dependent protein kinase or phosphorylase kinase (21). However, the role of the individual phosphorylation sites in the regulation of glycogen synthase by glucose-6-phosphate has not been extensively studied. Therefore, we compared the effects of mutations at regulatory  $\text{NH}_2$ -terminal (mutant 2) (Fig. 1) or COOH-terminal (mutant 3a) (Fig. 1) sites on the sensitivity of glycogen synthase to activation by glucose-6-phosphate. The concentration dependence of glucose-6-phosphate activation was determined in extracts of cell lines expressing these 2 mutant forms of glycogen synthase, and it was compared with that seen for wild-type enzymes. Wild-type glycogen synthase in cell extracts had a  $K_a$  for glucose-6-phosphate of  $\sim 1.6$  mmol/l

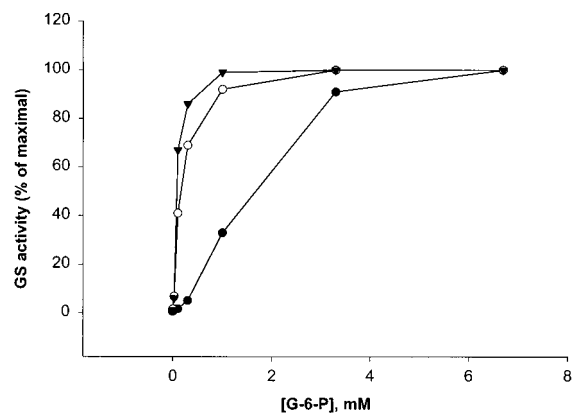


FIG. 3. Activation of wild-type and mutated glycogen synthase by glucose-6-phosphate. Activities of wild-type glycogen synthase (●) and mutant 2 (○) or mutant 3a (▼) were measured in the presence of various concentrations of glucose-6-phosphate, as indicated. Values represent an average of 2 independent experiments performed in duplicate.

(Fig. 3), a value close to the those previously reported for extensively phosphorylated protein (19,21). Mutation of either site 2 or site 3a significantly reduced the  $K_a$  values to 0.15 and 0.08 mmol/l, respectively (Fig. 3). Therefore, dephosphorylation at either the  $\text{NH}_2$ - or the COOH-terminus is capable of eliciting a substantial change in glucose-6-phosphate activation, which is likely to be an important aspect of glycogen synthase control in vivo.

## DISCUSSION

The results of this investigation lead us to conclude that insulin activation of glycogen synthase involves control of both  $\text{NH}_2$ - and COOH-terminal phosphorylation sites. This conclusion is consistent with earlier animal studies in which the phosphate content was measured directly (7–10). Additionally, our experimental approach allows us to reach 2 other conclusions. First, dephosphorylation of either the  $\text{NH}_2$ - or the COOH-terminus, as mimicked by appropriate phosphorylation site mutations, is sufficient to cause a significant increase in the sensitivity of the enzyme to glucose-6-phosphate. Thus, although insulin may normally control phosphorylation in both regions of the molecule, dephosphorylation at either region can significantly affect activity. Secondly, the results demonstrate that insulin-dependent dephosphorylation of site 3a in the COOH-terminus of glycogen synthase, which is the single most important regulatory site in the region, can occur in cell lines expressing mutant enzymes in which phosphorylation of site 3a cannot be mediated by GSK-3 (e.g. mutants 2,SA and 2,3b). Therefore, GSK-3 action is not essential for glycogen synthase activation by insulin, and GSK-3-independent mechanisms can operate. These mechanisms could be through control of protein phosphatases or other protein kinases that phosphorylate the COOH-terminus of glycogen synthase.

## ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (DK27221) and the American Diabetes Association.

## REFERENCES

1. Roach PJ: Liver glycogen synthase. In *The Enzymes*. Vol. 17A, 3rd ed. Boyer PD, Krebs EG, Eds. Orlando, FL, Academic Press, 1986, p. 499–539

2. Cohen P: Muscle glycogen synthase. In *The Enzymes*. Vol. 17A, 3rd ed. Boyer PD, Krebs EG, Eds. Orlando, FL, Academic Press, 1986, p. 461–497
3. Lawrence JC, Roach PJ: New insights into the role and mechanism of glycogen synthase activation by insulin. *Diabetes* 46:541–547, 1997
4. Skurat AV, Roach PJ: Phosphorylation of sites 3a and 3b (Ser<sup>640</sup> and Ser<sup>644</sup>) in the control of rabbit muscle glycogen synthase. *J Biol Chem* 270:12491–12497, 1995
5. Skurat AV, Wang Y, Roach PJ: Rabbit skeletal muscle glycogen synthase expressed in COS cells: identification of regulatory phosphorylation sites. *J Biol Chem* 269:25534–25542, 1994
6. Nakielny S, Campbell DG, Cohen P: The molecular mechanism by which adrenalin inhibits glycogen synthesis. *Eur J Biochem* 199:713–722, 1991
7. Parker PJ, Caudwell FB, Cohen P: Glycogen synthase from rabbit skeletal muscle: effect of insulin on the state of phosphorylation of the seven phosphoserine residues in vivo. *Eur J Biochem* 130:227–234, 1983
8. Lawrence JC Jr, Hiken JF, DePaoli-Roach AA, Roach PJ: Hormonal control of glycogen synthase in rat hemidiaphragms: effect of insulin and epinephrine on the distribution of phosphate between two cyanogen bromide fragments. *Eur J Biochem* 258:10710–10719, 1983
9. Smith RL, Roach PJ, Lawrence JC Jr: Insulin resistance in denervated skeletal muscle: inability of insulin to stimulate dephosphorylation of glycogen synthase in denervated rat epitrochlearis muscles. *J Biol Chem* 263:658–665, 1988
10. Lawrence JC Jr, Zhang J: Control of glycogen synthase and phosphorylase by amylin in rat skeletal muscle: hormonal effects on the phosphorylation of phosphorylase and on the distribution of phosphate in the synthase subunit. *J Biol Chem* 269:11595–11600, 1994
11. Cross DAE, Alessi DR, Cohen P, Andjelkovich M, Hemmings B: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–789, 1995
12. Picton C, Aitken A, Bilham T, Cohen P: Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle: organisation of the seven sites in the polypeptide chain. *Eur J Biochem* 124:37–45, 1982
13. DePaoli-Roach AA, Ahmad Z, Camici M, Lawrence JC Jr, Roach PJ: Multiple phosphorylation of rabbit skeletal muscle glycogen synthase: evidence for interactions among phosphorylation sites and the resolution of electrophoretically distinct forms of the subunit. *J Biol Chem* 258:10702–10709, 1983
14. Zhang W, DePaoli-Roach AA, Roach PJ: Mechanisms of multiple phosphorylation and inactivation of rabbit muscle glycogen synthase. *Arch Biochem Biophys* 304:219–225, 1993
15. Fiol CJ, Mahrenholz AM, Wang Y, Roeske RW, Roach PJ: Formation of protein kinase recognition sites by covalent modification of the substrate: molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J Biol Chem* 262:14042–14048, 1987
16. Skurat AV, Roach PJ: Multiple mechanisms for the phosphorylation of C-terminal regulatory sites in rabbit muscle glycogen synthase expressed in COS cells. *Biochem J* 313:45–50, 1996
17. Dent P, Lavoigne A, Nakielny S, Caudwell B, Watt P, Cohen P: The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature* 348:302–308, 1990
18. DePaoli-Roach AA, Suzuki Y, Lanner C, Zhang H, Yang J, Scrimgeour A, Lawrence JC: Glycogen metabolism in transgenic mice overexpressing or deficient in the muscle-specific glycogen/sarcoplasmic reticulum-associated phosphatase, PP1G (Abstract). *Diabetes* 48 (Suppl. 1):A25, 1999
19. Roach PJ, Takeda Y, Larner J: Rabbit skeletal muscle glycogen synthase. I. Relationship between phosphorylation state and kinetic properties. *J Biol Chem* 251:1913–1919, 1976
20. Roach PJ, Larner J: Rabbit skeletal muscle glycogen synthase. II. Enzyme phosphorylation state and effector concentrations as interacting control parameters. *J Biol Chem* 251:1920–1925, 1976
21. Embi N, Rylatt DB, Cohen P: Glycogen synthase kinase-3 from rabbit skeletal muscle: separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem* 107:519–527, 1980
22. Thomas JA, Schlender KK, Larner J: A rapid filter paper assay for UDP-glucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-<sup>14</sup>C-glucose. *Anal Biochem* 25:486–489, 1968
23. Guinovart JJ, Salavert A, Massague J, Ciudad CJ, Salsas E, Itarte E: Glycogen synthase: a new activity ratio expressing a high sensitivity to the phosphorylation state. *FEBS Lett* 106:284–288, 1979
24. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
25. Skurat AV, Cao Y, Roach PJ: Glucose control of rabbit skeletal muscle glycogenin expressed in COS cells. *J Biol Chem* 268:14701–14707, 1993
26. Wang Y, Roach PJ: Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3: dominant role of the phosphorylation of Ser-640 (site 3a). *J Biol Chem* 268:23876–23880, 1993