Glycogen synthase in rat adipocytes and skeletal muscle is phosphorylated on both serine and threonine

Jeffrey F. Hiken and John C. Lawrence, jr*

Department of Pharmacology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA

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Glycogen synthase is phosphorylated both in vivo and in vitro on multiple sites per subunit. All phosphorylations of the enzyme thus far identified occur on serines which are found in two cyanogen bromide fragments, denoted CB-1 and CB-2. We have immunoprecipitated [^{32}P]glycogen synthase from rat adipocytes and epitrochlearis muscles incubated with [^{32}P]phosphate. Phosphoamino acid analyses by two-dimensional electrophoresis after acid hydrolysis revealed no [^{32}P]phosphotyrosine, but significant levels of [^{32}P]phosphothreonine (6–14% of the [^{32}P](phosphoserine). The [^{32}P]phosphothreonine was recovered in the large CNBr-fragment (CB-2), indicative of a hitherto unknown phosphorylation site(s)

Glycogen synthase *Enzyme phosphorylation* *Phosphoserine* *Phosphothreonine* *Skeletal muscle* *Adipocytes*

1. INTRODUCTION

In skeletal muscle and fat cells, glycogen synthase catalyzes the normally rate-limiting step in glycogen synthesis from glucose. The activity of the enzyme is controlled by phosphorylation of multiple sites per subunit. Best characterized with respect to subunit structure is the rabbit skeletal muscle enzyme, which may be phosphorylated both in vitro and in vivo in at least 7 different phosphorylation sites [1,2]. Due largely to the work of Cohen et al. [3], the distribution of sites, and the sequences of amino acids surrounding the sites, have been identified. All of the phosphorylations thus far identified in the rabbit enzyme occur on serines found in only 2 CNBr fragments of the subunit.

Two general approaches have been used to investigate the phosphorylation of glycogen synthase in cells. One involves conventional purification of the enzyme, followed by chemical determinations of phosphate [4–7]. A second strategy is to label the enzyme by incubating tissues with [^{32}P]phosphate, and then to immunoprecipitate the [^{32}P]glycogen synthase [8–10]. An advantage of this approach is that phosphoamino acid analyses of the labeled enzymes can be readily performed. The results presented in this report indicate that glycogen synthase in both fat cells and skeletal muscle is phosphorylated to a significant level on threonine.

2. EXPERIMENTAL

Rat epididymal fat cells were isolated and incubated with [^{32}P]phosphate, and samples prepared for immunoprecipitation of glycogen synthase, exactly as described previously [10]. For skeletal muscle incubations, the epitrochlearis muscles from 80–100 g male rats (Sprague–Dawley) were incubated at resting length in Dulbecco's modified Eagle's medium modified to contain 0.1 mM phosphate. After 1 h, the muscles were transferred to fresh medium containing 0.1 mM [^{32}P]phosphate (2 mCi/ml) and incubated for 5 h at 37°C. The incubation was terminated by freezing the muscles in liquid nitrogen. The tissue was ground, and the powdered samples (~100 mg) were homogenized in 1 ml of buffer composed of 100 mM KF, 10 mM

* To whom correspondence concerning the manuscript should be addressed

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EDTA, 2 mM EGTA, 2 mM potassium phosphate, 10 mM benzamidine, and 50 mM Tris–HCl (pH 7.0 at 0°C). The homogenates were centrifuged at 10000 × *g* for 30 min, and supernatants were removed for immunoprecipitations.

[^{32}P]glycogen synthase was immunoprecipitated from cellular extracts (100 μl) as in [10], using antibodies prepared by immunizing guinea pigs with rabbit skeletal muscle glycogen synthase [9]. The immunoprecipitated enzymes were subjected to electrophoresis in the presence of sodium dodecyl sulfate (SDS) on slab gels (1.5 mm thick) formed with a linear gradient of acrylamide from 5 to 20%. When indicated, samples were cleaved with CNBr prior to electrophoresis. The bands corresponding to glycogen synthase or, CB-1 and CB-2, were sliced from the dried gels and incubated at 110°C for 2 h in 200 μl of 5.7 M HCl. After adding 5 ml of H_2O, the samples were centrifuged (2000 × *g* for 10 min) and the supernatants removed and evaporated to dryness by lyophilization. The samples were dissolved in H_2O and the phosphoamino acids were partially purified using Dowex AG1-X8 (Cl^- form) as in [11]. Samples were spotted onto cellulose thin layer sheets (no. 13255, Eastman Kodak) and phosphothreonine, phosphotyrosine, and phosphoserine were separated by two dimensional electrophoresis at pH 1.9 and pH 3.5 [11]. In some experiments, one dimensional electrophoresis at pH 1.9 was used to resolve phosphoserine from phosphotyrosine and phosphothreonine. For autoradiography, thin layer sheets were placed in X-ray casettes containing films (Kodak XAR-5) which were exposed at −80°C. The amounts of ^{32}P-labeled phosphoamino acids were quantitated by scintillation counting of appropriate spots of the thin layer sheets.

3. RESULTS AND DISCUSSION

In preliminary experiments, we found that immunoprecipitates of [^{32}P]glycogen synthase from rat adipocytes and skeletal muscle that had been incubated with [^{32}P]phosphate contained significant levels of [^{32}P]phosphothreonine. To be sure that the ^{32}P-labeled amino acid was associated with glycogen synthase, immunoprecipitated samples were subjected to electrophoresis on polyacrylamide gels in the presence of SDS. Under these conditions, the enzymes from both fat [10] and muscle [9] migrate as single bands of apparent M_r = 90000. The presence of [^{32}P]phosphothreonine in glycogen synthase was confirmed by analyses of the ^{32}P-labeled enzymes sliced from the gels (fig.1).

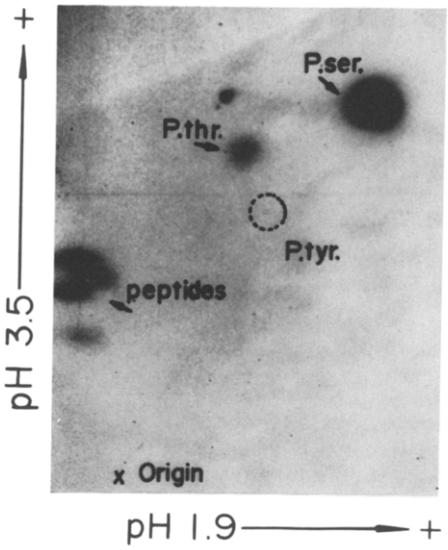

Fig.1. Separation of ^{32}P-labeled phosphoamino acids from rat skeletal muscle glycogen synthase by two-dimensional thin-layer electrophoresis. An autoradiogram of the thin-layer sheet is presented. The spots denoted P.ser and P.thr were coincident with ninhydrin staining of standards of phosphoserine and phosphothreonine, respectively. The dotted outline indicates the position of the phosphotyrosine standard where no radioactivity was detected.

Table 1

Relative proportions of ^{32}P-labeled phosphoamino acids in acid hydrolysates of adipocytes and skeletal muscle. The amounts of [^{32}P]phosphate found in each individual amino acid were divided by that found collectively in phosphotyrosine, phosphothreonine, and phosphoserine. Results are expressed as percentages, which from adipocytes are mean values (±SE) from 8 immunoprecipitations. Individual values from two experiments are presented for the muscle enzyme.

Enzyme source	Phosphorylated amino acid (%)		
	Tyrosine	Threonine	Serine
Adipocytes	0	6.4 ± 0.6	93.6 ± 0.6
Epitrochlearii	0	12.0, 11.4	88.0, 88.6

None was detected in corresponding slices of adjacent lanes of the gels to which samples processed with nonimmune IgG had been applied (not presented).

[^{32}P]phosphotyrosine was detected in glycogen synthase from neither fat nor muscle (table 1). In several experiments, the [^{32}P]phosphothreonine from the adipocyte enzyme has averaged ~7% of the [^{32}P]phosphoserine; however, the relative proportion of the ^{32}P-labeled phosphothreonine recovered from the muscle enzyme appears considerably higher (~14% of the phosphoserine).

Essentially all of the [^{32}P]phosphate is recovered in two fragments (CB-1 and CB-2) when enzyme immunoprecipitated from either skeletal muscle [9] or fat [10] is cleaved with CNBr, indicating that the [^{32}P]phosphothreonine must be found in one (or both) of these fragments. Phosphoamino acid analysis of the fragments from the fat cell enzyme

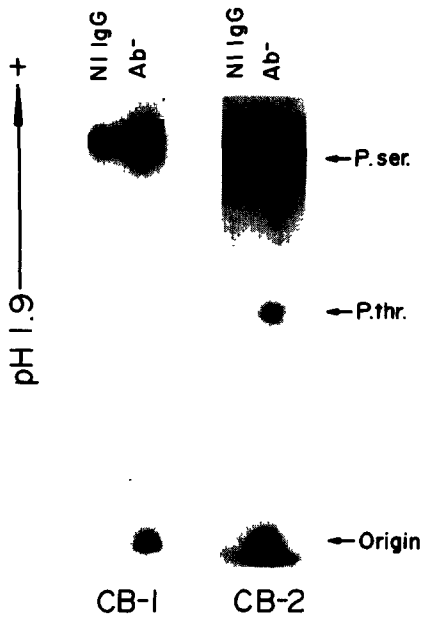

Fig.2. Recovery of [^{32}P]phosphothreonine in CB-2 of rat adipocyte glycogen synthase. Glycogen synthase was immunoprecipitated from fat cells using antibodies (Ab^-), and then cleaved with CNBr. Another sample was processed identically, except with nonimmune IgG (NI IgG) instead of Ab^-. After separation of fragments by polyacrylamide gel electrophoresis, samples were hydrolyzed, and one dimensional electrophoresis at pH 1.9 was used to resolve phosphoamino acids.

indicate that [^{32}P]phosphothreonine is present in CB-2, but not CB-1 (fig.2), providing evidence that threonines throughout the subunit are not randomly phosphorylated.

Glycogen synthase from rabbit muscle contains at least 7 serine phosphorylation sites [1,2]. Although detailed site analyses of the rat muscle and adipocyte enzymes have not been performed, these enzymes are also multiply phosphorylated [9,10]. Therefore, while the [^{32}P]phosphate recovered in phosphothreonine was considerably less than that in phosphoserine, it is in the range that might be expected of a single site. Furthermore, threonine in glycogen synthase in both muscle and fat cells was labeled by relatively short-term incubation with [^{32}P]phosphate, indicative of phosphate turnover in a metabolically active site(s).

Phosphorylation of purified rat skeletal muscle glycogen synthase is not well characterized. Even though it seems that none of the protein kinases thus far investigated phosphorylated threonine in rabbit skeletal muscle glycogen synthase, one or more kinases might phosphorylate threonine in the rat enzyme. It is conceivable that the rat enzyme contains a site that, save a threonine for serine substitution, is identical to a previously characterized site on the rabbit enzyme. Thus, demonstration of threonine phosphorylation suggests, but does not prove, that a completely novel site exists. The physiological significance of phosphothreonine phosphorylation of glycogen synthase remains to be determined. That this has been observed to occur in both muscle and fat cells suggests that it may be a widespread event, at least in rat tissues.

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REFERENCES

[1] Cohen, P. (1982) Nature 296, 613–620.
[2] Roach, P.J. (1981) Curr. Top. Cell. Regul. 20, 45–105.
[3] Picton, C., Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 124, 37–45.
[4] Roach, P.J., Roscll-Perez, M. and Larner, J. (1977) FEBS Lett. 80, 95–98.

[5] Uhing, R.J., Shikama, H. and Exton, J.H. (1981) FEBS Lett. 134, 185–188.
[6] Parker, P.J., Caudwell, B. and Cohen, P. (1983) Eur. J. Biochem. 130, 227–234.
[7] Sheorain, V.S., Juhl, H., Bass, M. and Soderling, T.R. (1984) J. Biol. Chem. 259, 7024–7030.
[8] McCullough, T.E. and Walsh, D.A. (1979) J. Biol. Chem. 254, 7336–7344.
[9] Lawrence, J.C., Jr., Hiken, J.F., DePaoli-Roach, A.A. and Roach, P.J. (1983) J. Biol. Chem. 258, 10710–10719.
[10] Lawrence, J.C., Jr. and James, C. (1984) J. Biol. Chem., in press.
[11] Cooper, J.A., Bartholomew, M.S. and Hunter, T. (1983) Met. Enzymol. 99, 387–402.