APPARENT PHOSPHORYLATION OF GLYCOGEN SYNTHASE IN MAMMALIAN CELLS LACKING CYCLIC AMP-DEPENDENT PROTEIN KINASE

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1. Introduction

The capacity of liver extracts to synthesize glycogen utilizing UDP-glucose as the glucose donor was first reported in [1]. Glycogen synthase (uridine diphosphate glucose: glycogen 4-α-glucosyl transferase, EC 2.4.1.11), the enzyme catalyzing this reaction, is a key enzyme in the regulation of glycogen synthesis in vivo [2,3]. Hormones promote the interconversion of synthase between activated dephosphorylated and deactivated phosphorylated forms [4,5]. Purified cyclic AMP-dependent protein kinase phosphorylated and deactivated purified glycogen synthase [6]. Thus it has been assumed that regulation of glycogen synthase involved interconversion between two kinetic forms: a D (or b) form, with activity largely dependent on the presence of the allosteric activator, glucose-6-P; and an I (or a) form, with activity essentially independent of glucose-6-P. This assumption led to the widespread use of the −/+ glucose-6-P activity ratio as a measure of the activation state of glycogen synthase.

However, the regulation of glycogen synthase is more complex (reviewed [3,7,8]). These reports indicate:

(i) Multiple purified kinases, both dependent and independent of cyclic AMP, can deactivate purified glycogen synthase by phosphorylating the synthase at distinct sites [3,9–19].

(ii) Hormones interconvert glycogen synthase among multiple glucose-6-P-dependent forms in intact tissues. This interconversion involves activities that resemble, kinetically, synthase phosphorylated by combinations of the purified kinases [20,21].

(iii) Chemical analysis of deactivated glycogen synthase from intact tissues has detected multiple phosphates on each subunit of the enzyme [3,22].

These data have led to the hypothesis that both cyclic AMP-dependent and -independent kinases phosphorylate and deactivate glycogen synthase in vivo. However, little is known about the precise function in intact tissue of these various kinases.

One approach for studying this problem is to use mutant cells with altered protein kinase activities. By studying cells that have lost a protein kinase, phenotypic characteristics that require the presence of that kinase can be established. Here, I present results obtained using mutants of S49 mouse lymphoma cells that lack cyclic AMP-dependent protein kinase (kin^-). The results lead to two conclusions:
to two conclusions:

1. Protein kinase (or kinases)* independent of cyclic AMP can substantially deactivate glycogen synthase in intact cells.
2. In growing S49 cells cyclic AMP-dependent protein kinase does not significantly contribute to the deactivation of glycogen synthase.

2. Experimental

S49 cells are a mouse lymphoma cell grown in suspension [23]. These cells are killed by drugs, hor-

* No evidence indicates that only one cyclic AMP-independent kinase is phosphorylating glycogen synthase in the reported experiments. Hereafter, the kinase activity will be expressed as singular, although more than one kinase may be involved.

Table 1
Characteristics of various clones of S49 cells

Clone	cAMP–dependent kinase phenotype	Characteristics [Ref.]
24.3.2	Wild type	Growth arrested and killed by cAMP [25,26]
211.12	Wild type	Growth arrested by cAMP [27]
24.6.1	No activity	No detected effects of cAMP [28–30]
U200.19	No activity	

mones, and toxins that increase intracellular cyclic AMP. The methods used to select cyclic AMP-resistant cells have been described [24]. The clonal cell lines used here are described and referenced in table 1. The clones of kin$^-$ cells were derived from independent selection experiments.

Glycogen synthase was extracted from S49 cells and assayed as follows: Cells, in exponential growth at 0.5–1.7 × 10^6 cells/ml, were harvested by centrifugation of 200 ml aliquots (180 × g, 5 min). The pelleted cells were washed with 75–100 ml ice-cold 5 mM Na-Hepes (pH 7.5) plus 150 mM NaCl and centrifuged once again. To ~0.5 ml pelleted cells was added 0.5 ml ice-cold buffer A or buffer B. Buffer A contained 50 mM Na-Hepes (pH 7.5), 2 × 10^3 U/ml Trasylol, 10 mM EDTA, 100 mM KF, 1.0 mM DTT, 1 mM PMSF. Buffer B was the same as buffer A, except 10 mM $MgCl_2$ replaced the EDTA, and KF was omitted. The cells were vortexed into the buffer and broken by sonication. The sonicates were centrifuged (12 000 × g, 15 min, 4°C) and the supernatants removed and stored on ice until used.

Assays of glycogen synthase were begun by the addition of 20–30 μl aliquots of supernatants to a reaction mix for 100 μl final vol. Final concentration of components contributed by the reaction mix were: [^{14}C]UDPG, 2.5 mM (50–100 × 10^3 cpm/tube); EDTA, 5 mM; Na-Hepes (pH 7.5), 25 mM; bovine liver glycogen, 15 mg/ml. Assays were run for 20 min at 30°C and the [^{14}C]glycogen purified and analyzed [31]. [^{14}C]Glycogen was directly proportional to time of assay and amount of added protein under these conditions. Kinetic constants were calculated as in [20]. Enzymatic activity is expressed as nmol ^{14}C incorporated into glycogen . min^{-1} . mg supernatant protein^{-1}.

Protein was determined by a modification [32] of the Lowry method [33]. [^{14}C]UDPG was from Amersham, bovine liver glycogen from PL Labs. and Trasylol from Mobay Chemical Corp. Other reagents were from Sigma.

3. Results

Glucose-6-P activated glycogen synthase in extracts of both wild type (wt) and kinase-minus (kin$^-$) S49 cells (table 2). The kinetic characteristics of these activations were remarkably similar.

(i) Activities in the absence of glucose-6-P were barely detectable.
(ii) The activation constants ($A_{0.5}$) were similar, and indicated the enzyme was multiply phosphorylated [3,13].
(iii) The glucose-6-P activation exhibited positively cooperative kinetics.

These results indicate that glycogen synthase is substantially and equally deactivated in both wt and kin$^-$ S49 cells. Because kin$^-$ cells lack cyclic AMP-

Table 2
Kinetic constants of the glucose-6-P activation of glycogen synthase from various clones of S49 cells.

Clone	Kinase phenotype	Glucose-6-P $A_{0.5}$	Glucose-6-P n_H	–/+ ratio	(N)
24.3.2	Wild type	3.3 ± 0.2	2.4 ± 0.1	≤ 0.02	(7)
211.12	Wild type	2.8 ± 0.3	2.3 ± 0.1	≤ 0.02	(3)
24.6.1	Kinase$^-$	2.7 ± 0.3	1.4 ± 0.1	≤ 0.03	(7)
U200.19	Kinase$^-$	3.2 ± 0	2.1 ± 0.1	≤ 0.02	(3)

Values are means ± SEM for (N) determinations

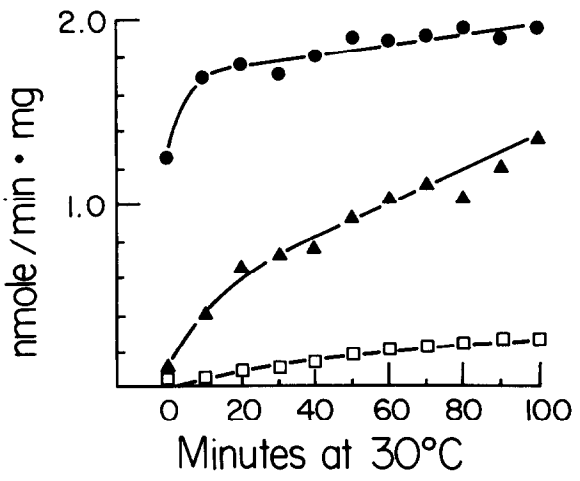

Fig.1. Activation of glycogen synthase in extracts of S49 cells. Supernatant extracts of wt (clone 24.3.2) were prepared in buffer B and incubated at 30°C. At the indicated times, aliquots were taken and immediately assayed for glycogen synthase activity at (□) 0, (▲) 0.4 and (●) 10 mM glucose-6-P. For details, see section 2.

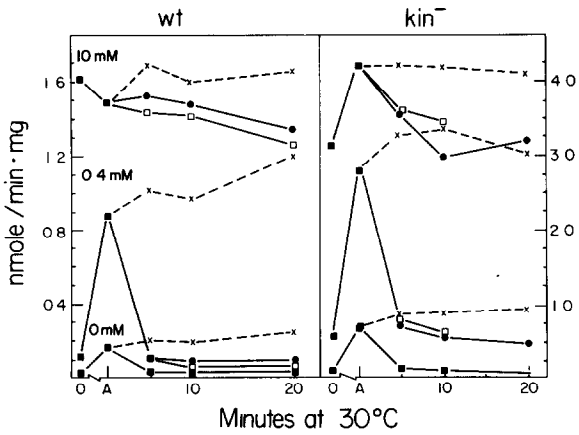

Fig.2. Deactivation of activated glycogen synthase. Supernatant extracts of both wt (clone 24.3.2) and kin⁻ (clone 24.6.1) cells were prepared in buffer B. Either before (■ at 0) or after (■ at A) activation, EDTA was added to aliquots and they were stored on ice. To other aliquots the following additions were made: (X) EDTA + ATP; (●) ATP; (□) ATP + cyclic AMP. After continuing the incubation at 30°C for the indicated times, aliquots were placed on ice and EDTA was added to the aliquots lacking EDTA. The aliquots were desalted by passage through a Sephadex G-25 column at 4°C equilibrated and eluted with 50 mM KF, 5 mM EDTA, 0.5 mM DTT, and 25 mM Na-Hepes (pH 7.5). Aliquots (30 μl) were assayed for glycogen synthase activity, as in section 2, at 0, 0.4 and 10 mM glucose-6-P.

dependent protein kinase [28,30], a plausible hypothesis is that a cyclic AMP-independent kinase is responsible for the deactivation. In order to test the hypothesis that phosphorylation caused enzyme deactivation, the enzyme was activated under conditions that promote dephosphorylation. The activation was then reversed by conditions that promote phosphorylation.

Activation of glycogen synthase occurred upon incubation of extracts at 30°C with Mg^{2+} in the absence of KF (fig.1). Activity increased when measured at 0, 0.4 and 10 mM glucose-6-P. Activity at 0.4 mM glucose-6-P rose far more than activity at either 0 or 10 mM. Thus, this activation is better considered as a substantial decrease in the $A_{0.5}$ (from ~3 mM to <0.4 mM) rather than an increase in either total or glucose-6-P-independent activity.

If dephosphorylation caused the activation just described, then phosphorylation of the enzyme should reverse the inactivation. Incubation of extracts containing activated glycogen synthase with ATP and Mg^{2+} led to enzyme deactivation. Addition of EDTA, to chelate the Mg^{2+} before addition of the ATP, prevented deactivation. Addition of cyclic AMP to the extract had no effect (fig.2). These results indicate that cyclic AMP-independent kinase deactivated the activated glycogen synthase in the cell extracts. In addition, incubating either wt or kin⁻ S49 cells with dibutyryl cyclic AMP under conditions known to activate cyclic AMP-dependent protein kinase in the wt cell [30] did not change the $A_{0.5}$ for glucose-6-P of the enzyme (not shown). These results suggest that cyclic AMP-independent kinase can substantially deactivate glycogen synthase in intact cells, and that in growing S49 cells, cyclic AMP-dependent kinase does not significantly contribute to the deactivation of glycogen synthase.

4. Discussion

Several reports indicate that purified protein kinases can phosphorylate purified substrates even though such phosphorylations may have no biological meaning [34–36]. Thus, although cyclic AMP-independent protein kinases can phosphorylate purified glycogen synthase [3,9–16,18,19], the biological significance of these phosphorylations may still be questioned. However, the results presented here indicate that phosphorylation of glycogen synthase by

cyclic AMP-independent protein kinase can occur in intact cells and warrants further study.

The −/+ glucose-6-P activity ratio fails to adequately describe the activation state of glycogen synthase [3,13,15,20,21]; the $A_{0.5}$ for glucose-6-P is reported instead. We have suggested [37] that the Hill coefficient (n_H) must also be reported in order to adequately describe the glucose-6-P activation of glycogen synthase. Specifically, we found that fasting increased the Hill coefficient of the glucose-6-P activation of adipose tissue glycogen synthase. When synthase from fat pads of fed and fasted rats were compared under certain conditions, only an increased Hill coefficient distinguished the glucose-6-P activation of the two preparations. It is interesting to note, therefore, that both wt and kin$^-$ cells yielded synthase preparations with elevated Hill coefficients. Thus cyclic AMP-dependent protein kinase is not required for positive cooperativity to be expressed. The structural characteristics responsible for the increased Hill coefficient have yet to be reported.

Finally, the kinetics of glucose-6-P activation of glycogen synthase from adipose tissue of fasted rats [37] and the enzyme from kin$^-$ clones of S49 cells (table 2) are strikingly similar. Both preparations have an elevated $A_{0.5}$ and Hill coefficient when compared to enzyme activity derived from fat pads from fed rats [37]. These results suggest that phosphorylations catalyzed by cyclic AMP-independent kinases are responsible for some of the alterations in glycogen metabolism caused by fasting.

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