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PHOSPHORYLATION AND INACTIVATION OF RAT HEPATOCYTE GLYCOGEN SYNTHASE BY PHORBOL ESTERS AND MEZEREIN

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Summary: Incubation of rat hepatocytes with active phorbol esters and mezerein provoked a decrease in glycogen synthase activity. After the incubation of [^{32}P] phosphate-labeled cells with these tumor promoters, an increase in the amount of ^{32}P bound to the immunoprecipitated enzyme was observed. The decrease in activity highly correlated with the phosphorylation in the smaller CNBr fragment (CB-1) and only at high concentration of the phorbol ester the increase in the phosphorylation of the larger CNBr fragment (CB-2) became significative. Tryptic degradation of CB-1 showed two phosphopeptides after isoelectro focusing analysis (pI 3.9 and pI 3.4) and only one of them (pI 3.9) increased its phosphorylation state after treatment of the cells.

These results indicate that the decrease in activity of glycogen synthase by phorbol esters and mezerein is a result of the phosphorylation of the enzyme and that a single site located in CB-1 is preferentially phosphorylated by these agents.

Glycogen synthase is regulated through phosphorylation and dephosphorylation reactions. Phosphorylation causes a decrease in the activity ratio of the enzyme (1,2). "In vitro", liver glycogen synthase can be phosphorylated at multiple sites by a set of protein kinases (3-8), including the calcium and phospholipid-dependent protein kinase, protein kinase C. All phosphorylation sites occur in only two CNBr fragments of the subunit (CB-2, 27-28 Kd and CB-1, 12-14 Kd). It has been recently shown that glycogen synthase from ^{32}P-labeled rat hepatocytes is also phosphorylated in these two

Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; 4β-PDD, 4β-phorbol-12,13-didecanoate; SDS, sodium dodecyl sulfate; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone.

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CNBr fragments. The phosphate content of the enzyme increases after the incubation of the cells with glycogenolytic hormones (7,9-11).

Phorbol esters may substitute for diacylglycerol in activating purified protein kinase C (12) and it is believed that protein kinase C acts as a receptor for these compounds (13). Mezerein, a second-stage tumor promoter (14,15) which has no diacylglycerol structure in its molecule, may also act on protein kinase C in the same way (16).

Some effects of forbol esters on glycogen metabolism have been recently described. In the perfused rat liver, Kimura et al. (17) showed that glycogenolysis could be stimulated by including TPA [1] in the perfusate, and Roach and Goldman reported that exposure of isolated rat hepatocytes to TPA resulted in a decrease in the glycogen synthase activity ratio (18).

In this paper we demonstrate that phorbol esters and mezerein cause a decrease in the activity of glycogen synthase by increasing its phosphorylation state. Evidence is presented that CB-1 region of the enzyme contains at least two phosphorylation sites, one of which becames highly phosphorylated after the stimulation of the cells with TPA.

MATERIALS AND METHODS

Isolation of ^{32}P-labeled glycogen synthase from rat hepatocytes. Cells were isolated and incubated with [^{32}P] phosphate as in (9). Effectors were dissolved in dimethyl sulfoxide. This solvent was added to control cells to achieve the same concentration (1% v/v) than in the treated cells.

At the end of the incubations cells were homogenized, the citosolic supernatant obtained, and ^{32}P-glycogen synthase immunoprecipitated using specific antibodies raised against rat liver glycogen synthase essentially as in (9). The washed immunopellets were dissolved in 200 μl of 50 mM β-mercaptoethanol, 70% formic acid solution. At this step, aliquots were taken to analyze the phosphorylation state of the enzyme subunit.

CNBr fragmentation of immunoprecipitated glycogen synthase. CNBr cleavage of the immunoprecipitated enzyme was carried out by incubation of the immunopellet in formic acid with CNBr (15 mg/ml) for 12 hours at room temperature. Samples were then evaporated and CNBr fragments resolved by electrophoresis using SDS-poliacrylamide slabs gels (6-20% gradient) essentially as in (19).

Tryptic analysis of glycogen synthase. After the identification of the ^{32}P -CNBr fragments of glycogen synthase by autoradiography, bands corresponding to CB-1 were scised, soaked in water, dried and incubated in 50 mM ammonium bicarbonate (pH 8.0) at 30° C with TPCK-treated trypsin (30 μg/ml) for six hours. The treatment was repeated until more than 90% of the radioactivity was eluted from the gel.

Isoelectric focusing of the tryptic peptides was carried out using 1 mm poliacrylamide gels. They were prepared from 25 ml

of a solution containing 4.375 ml of 40% (w/v) acrylamide, 4.375 ml of 1.08% (w/v) N,N-methylenebisacrylamide, 9 g of urea, 1.2 ml Ampholine pH2.5-4, 0.6 ml Pharmalyte pH 4-6.5, 0.05 ml of 85% phosphoric acid and 0.07 ml of 0.1 g/ml ammonium persulfate solution. 0.01 ml of N,N,N',N'-tetramethylethylenediamine were added to allow polimerization of the gel. The anode and catode solutions were 0.5 M sulfuric acid and 2% Pharmalyte pH 5-8, respectively. Isoelectric focusing was carried out at 3º C for 180-200 minutes, with a power setting of 18 watts and a maximun voltage of 1500 volts.

Autoradiograms were performed by placing the gels at -80º C in X-ray cassettes containing films (Kodak X-OMAT S) and intensifying screens. The intensity of the radioactive bands was measured with a densitometer equipped with an integrator.

Assays. Glycogen synthase activity was measured from hepatocyte extracts as in (20) and (21). Protein was determined by the biuret method.

RESULTS

Effects of phorbol esters and mezerein on the activity and phosphorylation state of glycogen synthase.

Isolated rat hepatocytes were pre-incubated with [^{32}P] phosphate for 45 minutes and then stimulated with the tumor promoters for 10 minutes. Glycogen synthase was rapidly isolated from either 10000 xg or 100000 xg supernatants by immunoprecipitation. The immunoprecipitated ^{32}P-enzyme was resolved by SDS-poliacrylamide gel electrophoresis. Analysis of the ^{32}P-labeled proteins from the gel by autoradiography showed a single ^{32}P-band corresponding to the 88Kd glycogen synthase subunit. It can be observed (Fig. 1)

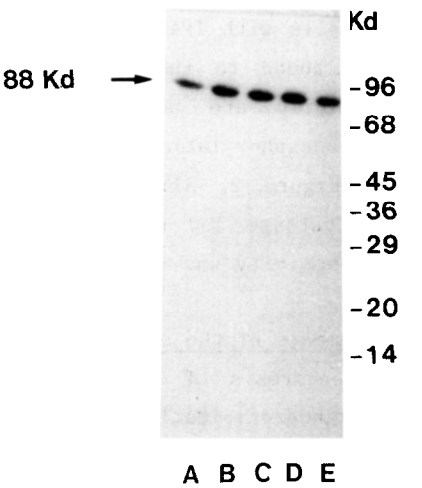
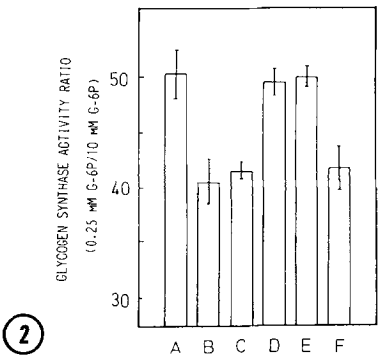


Figure 1. Electrophoretic analysis of ^{32}P-labeled glycogen synthase from A) Control cells. B) 100 ng/ml TPA. C) 100 ng/ml 4β-PDD. D) 100 ng/ml mezerein and E) 1000 ng/ml phorbol-treated cells. Immunoprecipitates of glycogen synthase were submited to electrophoresis in 6-20% gradient poliacrylamide gels and then autoradiograms were obtained. Molecular masses of standard proteins are shown on the right.



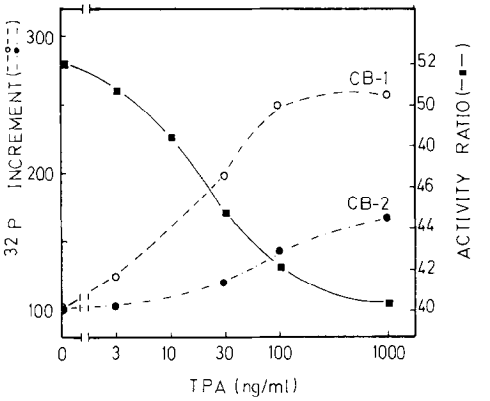


Figure 2. Effects of phorbol esters and mezerein on glycogen synthase activity. Hepatocyts were incubated with dimethyl sulfoxide (1% final concentration) or with the effectors for 10 minutes before extracts were prepared. Glycogen synthase low (0.25 mM) G-6P/high (10 mM) G-6P activity ratio was measured. A) Control cells. B)100 ng/ml TPA. C) 100 ng/ml 4β-PDD. D) 1000 ng/ml phorbol. E) 100 ng/ml 4α-PDD and F) 100 ng/ml mezerein-treated cells. Results are the mean $\pm$S.E.M. of at least four experiments.

Figure 3. Relationship beetwen glycogen synthase activity ratio and phosphorylation state of CB-1 and CB-2 fragments. Hepatocytes were incubated with increasing concentrations of TPA for 10 minutes in duplicate samples with and without [^{32}P]phoshate before extracts were prepared. Glycogen synthase low G-6P/high G-6P activity ratio was measured in extracts of cells not incubated with [^{32}P]phosphate. In extracts of ^{32}P-labeled cells, glycogen synthase was immunoprecipitated, cleaved with CNBr and subjected to electrophoresis. Autoradiograms of gels were scanned and the peaks corresponding to CB-1 and CB-2 were integrated. Results are expressed as percentage of the values from control cells.

that incubation of the cells with TPA, 4β-PDD and mezerein produced an increase in the ^{32}P bound to the enzyme. The parent compound phorbol and 4α-PDD, which are not biologically active, were uneffective in promoting phosphorylation of the enzyme.

As showed in Figure 2, all the tumor promoters able to phosphorylate glycogen synthase led to a decrease in the activity of the enzyme. However, activity was not changed by incubation with phorbol or 4α-PDD.

Analysis of the CNBr fragments of the enzyme.

SDS-gel electrophoresis of the CNBr cleaved ^{32}P-glycogen synthase isolated by immunoprecipitation yield two phosphopeptides, CB-2 (28 Kd) and CB-1 (14 Kd). TPA provoked a concentration-dependent increase in the radioactivity associated to CB-1 (Fig. 3). The decrease in activity of the enzyme perfectly correlated with the phosphorylation state of CB-1. A high decrease in the glycogen synthase activity ratio could be achieved without any significant increase in [^{32}P] phosphate bound to CB-2 and only at high

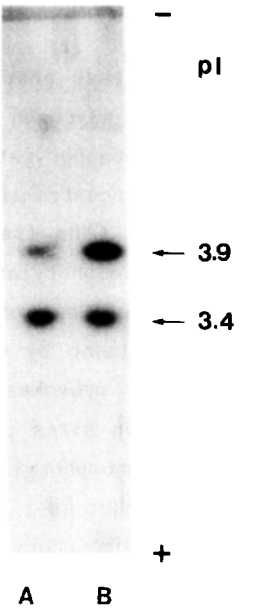


Figure 4. Isoelectro focusing of the tryptic peptides of CB-1. CB-1 bands were digested with trypsin before submission to isoelectro focusing and then autoradiograms were obtained. The apparent pI's are indicated on the right. A) Control cells. B) TPA-treated cells (100 ng/ml).

concentrations of TPA the [^{32}P] phosphate present in this fragment was significatively increased.

Tryptic analysis of CB-1

Fragments corresponding to CB-1 were subjected to exhaustive tryptic digestion followed by isoelectro focusing. Autoradiograms showed that CB-1 fragment contained at least two phosphorylation sites located into two phosphopeptides with apparent pI values of 3.9 (CB-1a) and 3.4 (CB-1b). Incubation of the cells with TPA (100 ng/ml) for 10 minutes did not change the level of phosphorylation of CB-1b. However, a pronounce increase in the ^{32}P bound to CB-1a was observed (Fig. 4 and Table 1).

TABLE 1

	CB-1a	CB-1b	CB-1b / CB-1a
CONTROL	100	100	2.5 ± 0.4
TPA (100 ng/ml)	340 ± 57	103 ± 21	0.9 ± 0.1

Autoradiograms of the gels were scanned and the peaks corresponding to the two tryptic fragments derived from CB-1 were integrated and expressed as percentage from control cells. Results are the mean ± S.E.M. of four experiments.

DISCUSSION

Our results demonstrate that phorbol esters and mezerein decrease the activity of hepatic glycogen synthase by increasing its phosphorylation state. This phosphorylation is highly specific. Firstly, these agents at low concentration specifically provoke the phosphorylation of the smaller CNBr fragment of the enzyme (CB-1) and the level of this phosphorylation closely correlates with the decrease in the glycogen synthase activity ratio. Secondly, upon further analysis of CB-1 fragment by tryptic digestion, it has been shown that TPA specifically provokes the phosphorylation of only one of the two phosphorylation sites present in this fragment, without affecting the degree of phosphorylation of the second site at all. In fact, the observation that CB-1 fragment of the rat liver enzyme contains two sites of phosphorylation is an important additional conclusion of this paper. It is also worth to note that, in this system, mezerein is able to elicit the same metabolic effects as active phorbol esters, probably as a result of a common mechanism of action.

Since phorbol esters and mezerein are known to activate protein kinase C (12,16) it is logical to assume that this kinase is responsible for the phosphorylation observed. In that sense, it is known that "in vitro" protein kinase C phosphorylates rat liver glycogen synthase only at CB-1 (4,7), the same fragment which is preferentially phosphorylated by phorbol esters and mezerein in rat hepatocytes. It must be stressed that only those phorbol derivatives acting as tumor promoters, which are precisely those able to activate "in vitro" protein kinase C (12), are also capable to produce the phosphorylation of rat hepatocyte glycogen synthase. This provides an additional argument for the involvement of protein kinase C in the phosphorylation of the enzyme. Therefore, our results suggest that protein kinase C may play an important role in the regulation of glycogen synthesis in liver cells.

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