Phosphorylation of histones and non-histone nuclear proteins in liver cells stimulated by glucagon and cyclic AMP

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Phosphorylation of a characteristic subset of nuclear proteins is increased in rat liver cells stimulated with glucagon. Regulated proteins include histones H1 and H3, an HMG 14-like protein and a previously unidentified 23-kDa basic protein. The effect of glucagon is mimicked by forskolin and exogenous cAMP. Insulin and dexamethasone have no effect. In a cell-free system containing purified hepatocyte nuclei, addition of cAMP-dependent protein kinase results in phosphorylation of histone H3, an HMG 14-like protein and a 23-kDa basic protein similar or identical to the protein phosphorylated in vivo.

Glucagon *cAMP* *Hepatocyte* *Protein phosphorylation* *Chromatin* *Histone*

1. INTRODUCTION

Glucagon plays a key role in the regulation of liver cell function. Most of the hormone effects appear to depend on activation of A-kinase and subsequent phosphorylation of various proteins [1,2]. Several key enzymes of intermediary metabolism have been identified among cytosolic proteins phosphorylated in response to glucagon in the hepatocyte [1]. These enzymes serve as substrates for A-kinase in vitro and their activities are affected by phosphorylation in a way which explains the acute effects of glucagon on the concerned metabolic pathways [3]. Glucagon, on the other hand, has additional effects which do not simply reflect the covalent modification of regulatory enzymes of metabolism. The hormone is known to regulate the rate of synthesis of specific enzymes and may also play some role in triggering hepatocyte proliferation [4,5]. Whether the activation of A-kinase and the phosphorylation of as yet unidentified proteins are instrumental in such processes is an open question. To approach this issue, it was of interest to look for proteins in the nucleus of the hepatocyte which might be phosphorylated under the influence of glucagon. Herein, we describe a hitherto unrecognized pattern of nuclear protein phosphorylation in cultured liver cells stimulated with glucagon and related effectors.

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Abbreviations: A-kinase, cAMP-dependent protein kinase; HMG proteins, high mobility group proteins; NbS_2, 5′,5-dithiobis(2-nitrobenzoate); IBMX, 3-isobutyl-1-methylxanthine

2. MATERIALS AND METHODS

Hepatocytes from fasted, glucose-refed rats were isolated and cultured in suspension as in [4]. After 3.5 h in culture, cells were washed and transferred to a low phosphate-medium (0.1 mM potassium phosphate) for labeling with [^{32}P]orthophosphate. Routinely, 120×10^6 cells were labeled for 1.5 h in 40 ml of medium containing 110 μCi [^{32}P]orthophosphate/ml. Thereafter, the

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cell suspension was distributed to several vials (15 $\times$ 10^6 cells/vial) containing various hormones or effectors. Incubation with the effectors was for 30 min, in the continued presence of [^{32}P]orthophosphate. The incubation was terminated by centrifuging the cells through pads of ice-cold Percoll (Pharmacia) solution (density 1.06) at 180 $\times$ g_{max} for 1 min.

Nuclei stripped of their envelope were isolated from labeled cells by a low ionic strength-dependent procedure basically as in [6], using two rounds of centrifugation through sucrose. All solutions were kept at 4°C and contained 0.1 mM NbS_2 as inhibitor of protein phosphatases [7]. The final nuclear pellet was dispersed by sonication in 10 mM Tris–HCl, 20 mM EDTA and 0.1 mM NbS_2, pH 7.0. Sulfuric acid was added to a final concentration of 0.2 M for extraction of total acid-soluble proteins. In some cases, histone H1 and the HMG proteins were extracted from parallel samples with $HClO_4$ at a final concentration of 0.74 M.

In vitro phosphorylation of nuclear proteins was investigated in a cell-free system consisting of purified nuclei and exogenous A-kinase. Nuclei for these experiments were obtained from hepatocytes which were cultured for 5 h in hormone-free medium. The isolation of nuclei was essentially by Method II in [8]. Purified nuclei were used immediately for the phosphorylation reaction. The latter was performed at 4°C for 1 h in a solution containing 0.25 M sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.6 mM [γ-^{32}P]ATP (spec. act. 0.05 Ci/mmol) and 2 μM cAMP in a total volume of 1 ml containing 150 $\times$ 10^6 nuclei, in the presence or absence of 180 μg/ml of partially purified type II A-kinase from bovine heart (Sigma). The nuclei were maintained in suspension by mixing with a glass rod. The incubation was terminated by pelleting the nuclei and washing them twice in cold buffer. Nuclei were then disrupted by sonication in 10 mM Tris–HCl, 1.5 mM EDTA, pH 7.0, and acid-soluble proteins were extracted in 0.2 M H_2SO_4.

After precipitation with acetone or trichloroacetic acid, acid-soluble nuclear proteins were analyzed by two-dimensional gel electrophoresis. Separation in the first dimension was accomplished by acid–urea–Triton gel electrophoresis as in [9], using cylindrical acrylamide gels containing 0.85 M CH_3COOH, 5 M urea and 6 mM Triton X-100. Electrophoresis in the second dimension was performed in SDS-polyacrylamide slab gels as in [10], using 15% polyacrylamide separation gels. The first-dimension gels were equilibrated for 1 h in the sample buffer described in [10] and fused to the slabs with an agarose bridge. Dried gels were autoradiographed at -70°C on preflashed Kodak X-Omat SO-282 films, using intensifying screens.

3. RESULTS

3.1. *Hormonal regulation of nuclear protein phosphorylation in liver cells*

Hormonal effects on nuclear protein phosphorylation were investigated in rat liver cells in short-term suspension culture. Glucagon or other effectors were added to the cells after a preincubation of 5 h in hormone-free medium. The final 1.5 h of preincubation were conducted in the presence of [^{32}P]orthophosphate to label cellular ATP, the phosphate donor in protein phosphorylation reactions.

Fig.1 depicts the pattern of acid-soluble nuclear proteins phosphorylated in control cells (A) and in cells exposed to 2 $\times$ 10^{-7} M glucagon for 30 min (B). The phosphorylation of histone H1 appeared enhanced in treated cells, as expected from earlier data obtained with the intact animal [11]. Moreover, glucagon stimulated the phosphorylation of three other proteins which were not previously known to be hormonally regulated. These proteins are: (i) histone H3, including the 3 variants of the protein; (ii) a protein (arrow in fig.1) with apparent M_r 23000 according to its migration in SDS-polyacrylamide gels. We have termed it 23-K basic regulated protein (23-K BRP); (iii) one of 3 closely related polypeptides with electrophoretic mobilities characteristic of HMG 14 protein in acid–urea–Triton gels as well as SDS gels. The separation of these polypeptides by one-dimensional acid–urea–Triton gel electrophoresis of perchloric acid nuclear extracts is shown in fig.2. As may be seen, only one of the polypeptides, which migrates with intermediate mobility, has its phosphorylation state affected by glucagon.

We next assessed the ability of exogenous cAMP and of forskolin to mimic the effects of glucagon. Insulin and dexamethasone were also tested in

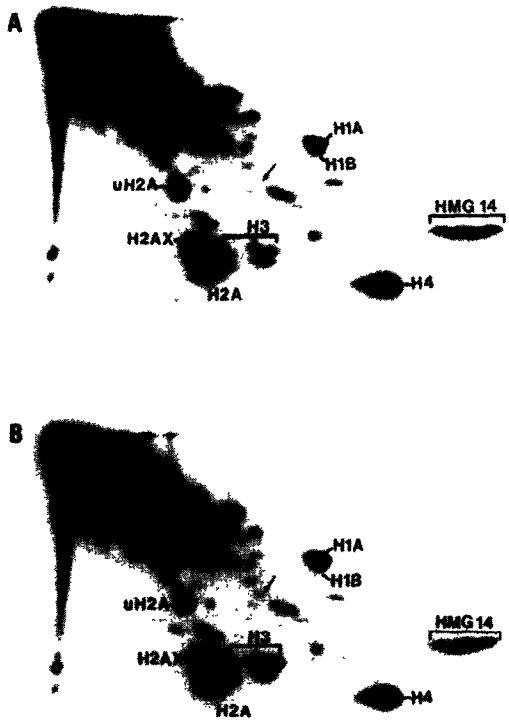

Fig.1. Autoradiographs showing the effect of glucagon on phosphorylation of acid-soluble nuclear proteins in hepatocytes. (A) Pattern of phosphorylated proteins in control cells. (B) Pattern of phosphorylated proteins in cells treated with 2×10^{-7} M glucagon for 30 min. Histones were identified according to their electrophoretic mobilities in the first dimension, based on data in [9]. The HMG proteins were identified on the basis of electrophoresis data published in [12]. The nomenclature for H2A variants is as proposed in [13].

these experiments. Radioactive phosphate incorporation into selected proteins was measured by liquid scintillation spectrometry after excising the corresponding spots from stained gels similar to those of fig.1. To cancel minor variations in [^{32}P]phosphate uptake and nuclear protein yield between samples, the data were expressed as relative incorporation values, i.e., ratios of radioactivity in the protein of interest to the radioactivity of histone H4 in the same sample. Histone H4 was chosen as benchmark protein because it did not seem to be affected by any of the hormone treatments. As shown in table 1, cAMP and forskolin as well as glucagon stimuated the phosphorylation of histone H1, H3 and of the HMG 14 protein. These data support the view that cAMP is the intracellular mediator for nuclear protein phosphorylation. The increase over baseline phosphorylation was largest in H3 and smallest in the HMG 14 polypeptides. The latter were insufficiently resolved in the present gels to allow individual radioactivity measurements. The total radioactivity in the HMG 14 area was nevertheless determined to ascertain the reproducibility of hormonal effects. In every experiment, phosphorylation of the 3 polypeptides considered as a whole was increased in cells challenged with glucagon, cAMP or forskolin. Neither insulin nor dexamethasone had any effect on the phosphorylation of histones H1, H3 or the HMG 14 proteins. Moreover, no other protein resolved in the two-dimensional gel electrophoresis system appeared to be affected by these hormones. The addition of dexamethasone simultaneously with glucagon did not result in an enhancement of the effects seen with the latter hormone alone. In all the experiments summarized in table 1, phosphorylation of 23-K BRP was analyzed by autoradiography. This protein was consistently phosphorylated in response to glucagon, cAMP or forskolin, but not after exposure of the cells to insulin or dexamethasone.

Time-course experiments (not shown) demonstrated that phosphorylation of histone H3 is maximal within 3 min of glucagon addition and is maintained at a plateau for at least 2 h in the presence of the hormone.

3.2. *Cell-free phosphorylation of nuclear proteins*

In view of the evidence for the participation of the cAMP system in the phosphorylation of nuclear proteins in the intact cell, it was of interest to examine in vitro protein phosphorylation by exogenous A-kinase in isolated nuclei. Nuclei for these experiments were isolated from unstimulated liver cells. Cell-free phosphorylation was accomplished in the presence of [γ-^{32}P]ATP and cAMP, with or without added A-kinase. A typical autoradiogram of proteins phosphorylated in control nuclei, i.e., by endogenous nuclear protein kinases, is shown in fig.3A and the corresponding pattern in nuclei incubated with exogenous A-kinase in fig.3B. The changes due to the added

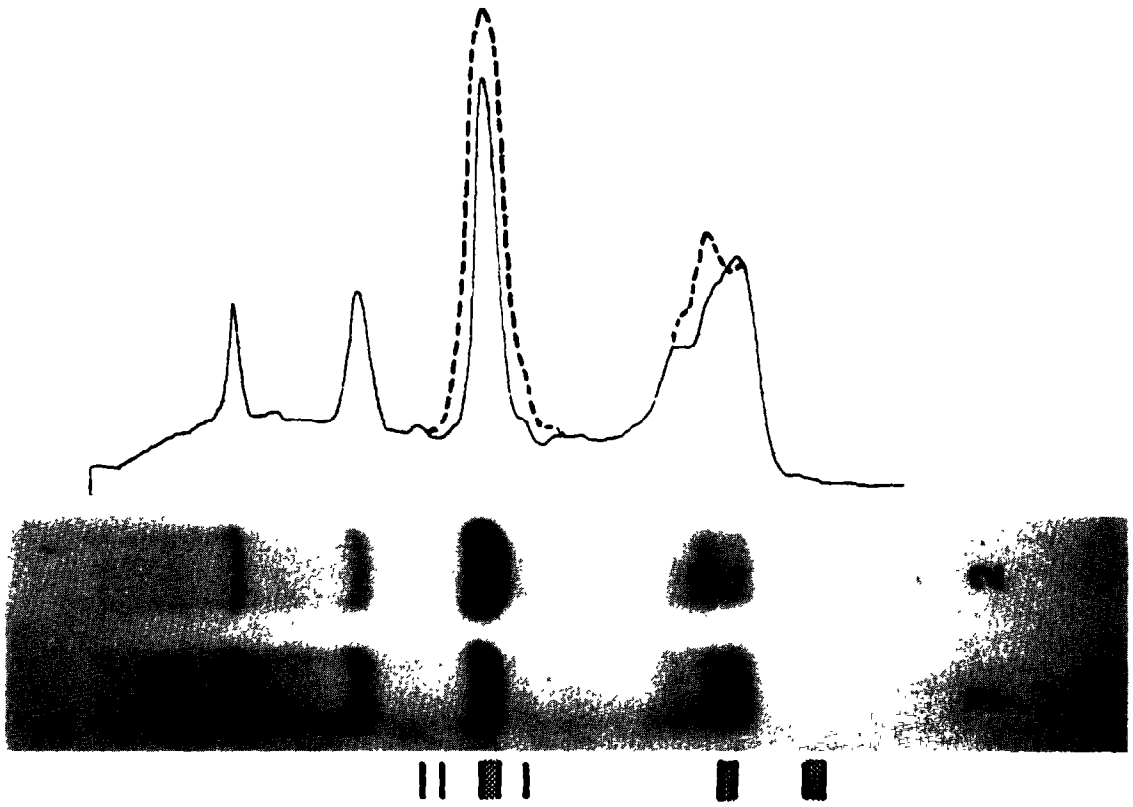

Fig.2. Separation of perchloric acid-soluble nuclear proteins by one-dimensional acid–urea–Triton gel electrophoresis. Track 1: autoradiograph showing phosphoproteins in control cells. Track 2: phosphoproteins in glucagon-treated cells. Hormone treatment was as in fig.1. Marks on the left indicate the position in the gel of stained bands corresponding, from top to bottom, to HMG1, HMG2, H1, H1°, HMG14 and HMG17. Traces on the right are densitometer scans of track 1 (——) and track 2 (– – –).

Table 1

Hormonal regulation of nuclear protein phosphorylation in hepatocytes

Hormone treatment	^{32}P incorporation in			
	H1	H3	HMG14	H2A
None	16.3 ± 1.2	13.4 ± 4.1	22.0 ± 4.0	574 ± 39
Glucagon, 2×10^{-7} M	23.0 ± 1.0	31.9 ± 2.4	27.7 ± 4.7	568 ± 39
cAMP, 3×10^{-4} M + IBMX, 5×10^{-4} M	32.8 ± 6.0	53.6 ± 7.3	33.3 ± 7.2	590 ± 18
Forskolin, 1.5×10^{-4} M	18.8 ± 1.3	21.3 ± 2.9	26.2 ± 6.9	591 ± 35
Insulin, 10^{-8} M	16.2 ± 0.7	12.8 ± 2.2	23.0 ± 4.1	590 ± 48
Dexamethasone, 10^{-8} M	15.4 ± 0.1	12.0 ± 5.1	23.0 ± 6.0	566 ± 8
Dexamethasone + glucagon	24.3 ± 0.4	32.4 ± 5.4	30.0 ± 5.6	607 ± 3

See section 2 for cell culture and labeling conditions. Treatment with effectors was for 30 min. Phosphate incorporation in proteins is expressed in percent, relative to the incorporation in histone H4 serving as reference protein. Incorporation in H1 is the sum of incorporations in components H1A and H1B. Incorporation in H3 is the sum of incorporations in variants 1,2 and 3. Histone H2A is included as an example of hormone unresponsive protein. Data are means ± SD of at least 3 experiments

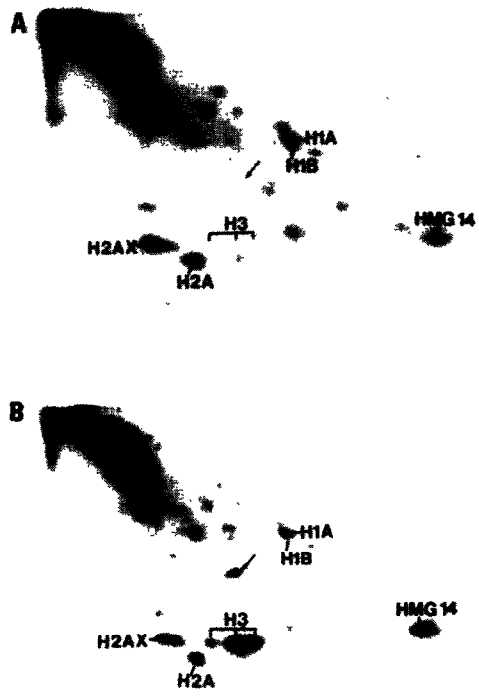

Fig.3. Autoradiographs showing in vitro phosphorylation of proteins by exogenous cAMP-dependent protein kinase in isolated nuclei. (A) Pattern of proteins phosphorylated in nuclei without added A-kinase. (B) Pattern of proteins phosphorylated in the presence of A-kinase.

kinase are: (i) phosphorylation of histone H3; (ii) phosphorylation of a protein with an apparent M_r of 23000 (arrow in fig.3). Based on electrophoretic mobilities in acid–urea–Triton gels, as well as in SDS gels, this protein appears to be the counterpart of the 23-K BRP identified in intact cells; (iii) phosphorylation of HMG 14 protein. These changes are strikingly similar to those occurring in vivo in intact cells upon stimulation with glucagon. The hormone-dependent phosphorylation of histone H1, however, was not reproduced in the present cell-free experiments. The effects of A-kinase in the cell-free system were suppressed when incubation was performed in the presence of the heat-stable protein inhibitor of the kinase (not shown).

4. DISCUSSION

The authors in [11] showed previously that histone H1 is phosphorylated in rat liver following glucagon administration. Here, we demonstrate the phosphorylation of several additional nuclear proteins in cultured liver cells challenged with glucagon, forskolin or cAMP. Besides histone H1, regulated proteins include histone H3, an HMG 14-like protein and a basic protein with apparent M_r 23000. The in vivo pattern of protein phosphorylation is quite faithfully reproduced in vitro when nuclei from unstimulated hepatocytes are incubated with A-kinase.

The present results are consistent with recent data on the phosphorylation of histone H3 and HMG 14 in other systems. As shown in [14], histone H3 is the only core histone phosphorylated by A-kinase in cell-free systems containing chromatin or nucleosome cores. A unique phosphorylation site (Ser 10) appears to be present in the molecule [14]. Presumably, H3 was phosphorylated at this site in our experiments, both in vivo and in vitro. Authors in [12] recently reported the electrophoretic separation of 4 distinct HMG 14 polypeptides from HeLa cells. Only one of these, termed β1, served as substrate for A-kinase. The hormonally regulated HMG 14 polypeptide of rat liver cells appears similar to the β1 protein of these authors.

The 23-K BRP phosphorylated in response to glucagon may be of special interest, in view of its low abundance and, therefore, of its potential regulatory significance. The amount of the protein in the nucleus appears to be 5–10-fold less than that of HMG 1, as estimated from its staining in electrophoretic gels of total nuclear protein. The 23-K BRP is not related to the HMG group, since it is not extractable in perchloric acid. Suggestive evidence indicates that the same protein can be phosphorylated in vitro in a nuclear cell-free system, either by A-kinase directly or by a kinase cascade. Our recent experiments suggest that the 23-K protein phosphorylated in vitro by A-kinase is associated with the nuclear matrix. Interestingly, a hormonally regulated nuclear phosphoprotein which migrates in acid–urea–Triton gels similar to the liver 23-K BRP has been identified in GH_4 pituitary cells. The pituitary protein, however, appears to have a higher M_r as estimated by SDS-

polyacrylamide gel electrophoresis [15,16].

The biological significance of the hormone-dependent phosphorylation of nuclear proteins is unknown. Previous work in this laboratory has shown that liver cells in short-term suspension culture respond to glucagon or cAMP by a rapid induction of P-enolpyruvate carboxykinase (GTP) synthesis, as a result of specific mRNA accumulation [4]. The cAMP-mediated build-up of P-enolpyruvate carboxykinase mRNA depends at least in part on a stimulation of gene transcription [17]. The phosphorylation of histone H1, histone H3 and the HMG 14 protein would be expected to result in rather widespread alterations in chromatin conformation. Whether and how such changes might account for specific effects on the transcription of one or a few genes is a matter for speculation. Another intriguing feature emerging from this work is the similarity between the pattern of nuclear protein phosphorylation elicited by glucagon in liver cells and changes occurring prior to mitosis in various cell lines [18,19]. Finally, it should be pointed out that the cAMP-mediated phosphorylation of histone H1, histone H3 and the HMG 14 protein is not restricted to the liver cell, as evidenced by similar findings [20] in the thyroid gland stimulated with thyrotropin.

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