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A NEW cAMP INDEPENDENT PROTEIN KINASE TIGHTLY BOUND TO DNA, IN RAT LIVER NUCLEI

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SUMMARY. A protein kinase has been characterized among the proteins tightly bound to DNA. It is not extracted with 1 M NaCl and is released by extensive DNase I digestion. This enzyme is able to phosphorylate nucleosomal histones, essentially H2B and H3, and several non-histone proteins associated with DNA, on serine residue(s). It does not phosphorylate protamine, casein, phosvitin and the chromosomal non-histone proteins extracted with 1 M NaCl and is cAMP independent. This protein kinase can be distinguished from the previously described enzymes.

Chromatin protein phosphorylation has been implicated in the regulation of gene expression and of cell growth. Two types of nuclear protein kinases have been characterized in cell nuclei. One type, which includes protein kinases NI and NII, uses acidic proteins, including chromosomal non-histone proteins and exogenous proteins as casein and phosvitin as substrates (1-7). The other type uses histones as substrates. Two histone kinases specific of H3, one cAMP independent (8), another cAMP dependent (9), have been characterized in calf thymus. The last enzyme has been shown to loose partly its specificity when incubated with a mixture of histones, it is then able to phosphorylate in addition histones H2B and H4 (9). An histone kinase specific of H1 has been shown to trigger mitosis (10). Another histone kinase characteristic of H1 has been characterized in plasmocytoma cells (11). Phosphorylation of histones H1 and H3 varies according to the phase of the cell cycle (12,13).

In our attempt to purify proteins with high affinity for DNA, we have found a new protein kinase which is able to phosphorylate nucleosomal histones, essentially H3 and H2B and a discrete set of DNA-bound non-

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histone proteins. This enzyme, which is cAMP independent, is unable to phosphorylate phosvitin, casein, protamine and the chromosomal non-histone proteins extracted with 1 M NaCl.

MATERIALS AND METHODS

Preparation of DNA bound proteins. Nuclei were isolated from liver of Wistar male rats weighing 180-220 g, fasted for 36 h and purified according to Chauveau *et al.* (14). The nuclei were washed once with 10 mM Tris-HCl (pH 7.5), 3 mM $CaCl_2$, 0.2 % NP40 and twice with the same buffer but without NP40. They were suspended in 1 M NaCl, 10 mM Tris-HCl (pH 7.5) and stirred gently overnight. The insoluble material was collected by 90 min of centrifugation at 40 000xg in a R30 Beckman rotor. The pellet was washed with 1 M NaCl, 10 mM Tris-HCl (pH 7.5). It includes DNA, histones and several non-histone proteins, tightly bound to DNA. The proteins were released by digestion with 2.5 mg DNase I (Sigma), 5 mM $MgCl_2$ for 6 h at 20°C. The suspension was centrifuged and the supernatant was dialyzed against 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$. It will be referred to as DNA-bound proteins or DBP.

Determination of the protein kinase activity of DBP. The incubation medium included in a final 250 µl volume for each assay : 100 µl of DBP solution containing 100 µg of proteins, 10 mM $MgCl_2$ (which was found optimal), 5 µCi of [γ-^{32}P] ATP (specific activity : 30 Ci/mmol - Amersham, U.K.), 10 mM Tris-HCl (pH 7.5). The incubations were carried out for 60 min at 37°C. The proteins were precipitated with 1 ml of 33 % trichloroacetic acid. The precipitates were collected on Millipore HAWP 0.45 µ filters and washed with 10 ml of 25 % trichloroacetic acid. The filters were transferred in 5 ml of Unisolve (Koch Light Lab.) and the radioactivities measured in a SL 36 Intertechnique spectrometer.

Electrophoreses. Electrophoreses were performed in SDS-7.5 to 20 % gradient polyacrylamide gels according to Laemmli (15). After migration, the gels were stained for 15 min with Coomassie Blue and destained with 30 % methanol, 10 % acetic acid and dried.
Radioautographies were obtained with NS 2T Kodak films. NEN [^{14}C] labelled and BioRad molecular weight markers were used.

Analysis of phosphoaminoacids. Phosphoproteins were analyzed for [^{32}P] phosphoaminoacids. The proteins were hydrolyzed and the aminoacids were separated by electrophoresis-chromatography on cellulose thin layer plates according to Hunter and Sefton (16).

RESULTS

Presence of a protein kinase in the DNA-bound protein fraction. When 100 µg of DBP were incubated in the presence of [γ-^{32}P] ATP, a transfer of ^{32}P into trichloroacetic material was observed. The time course of phosphate transfer, measured at 20°C (Fig.1), strongly suggests that this transfer is enzyme dependent and that a protein kinase is present among the DNA-bound proteins. The specific activity of this enzyme is 6.4 pmol/min/mg DBP.

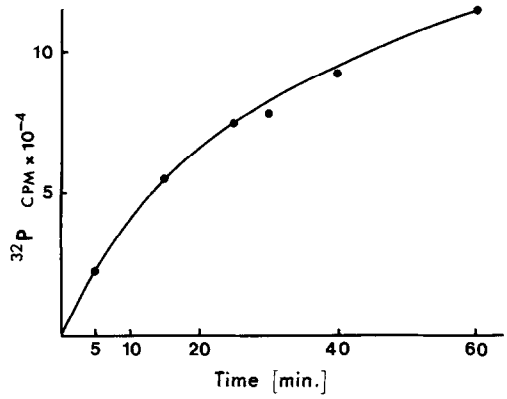

Figure 1. Time course of DNA bound protein phosphorylation. DBP were incubated at 20°C in the presence of [γ-^{32}P] ATP for various lengths of time. Samples containing 100 µg of DBP were taken for each assay. The radioactivity of the trichloroacetic precipitates was measured on millipore filters.

Substrate specificity of the enzyme. An aliquot of the incubation medium was dissolved in the Laemmli sample buffer and submitted to a SDS-polyacrylamide gel electrophoresis followed by a radioautography. Fig.2 shows that the major substrates are histones H3 and H2B. Histones H2A and H4 were phosphorylated to a much lesser extent. When 100 µg of total histones were added to the incubation mixture, additional 2.4 pmol/min/mg DBP of phosphate were incorporated ; in the presence of 100 µg of histone H2B, additional 4.3 pmol/min/mg DBP of phosphate were incorporated. When incubations were carried out in the absence of added proteins, approximately 10 non-histone DBP with molecular weights ranging from 20 000 to 75 000 were found radioactive. Chromosomal non-histone proteins extracted with 1 M NaCl, in which protein kinases NI and HII have been either inactivated by heating for 10 min at 55°C or removed by chromatography, were not phosphorylated when added to the incubation medium. None of the following proteins : phosvitin, casein, protamine and histone H1 were phosphorylated under the same conditions. Fig.3 shows that the endogenous substrates were phosphorylated strictly on serine residues.

Some properties of the DBP protein kinase. The protein kinase described in this paper is not affected by cAMP at concentrations from 10^{-6} to 10^{-5}M. ADP and GTP, even at high concentrations, did not inhibit phosphate transfer

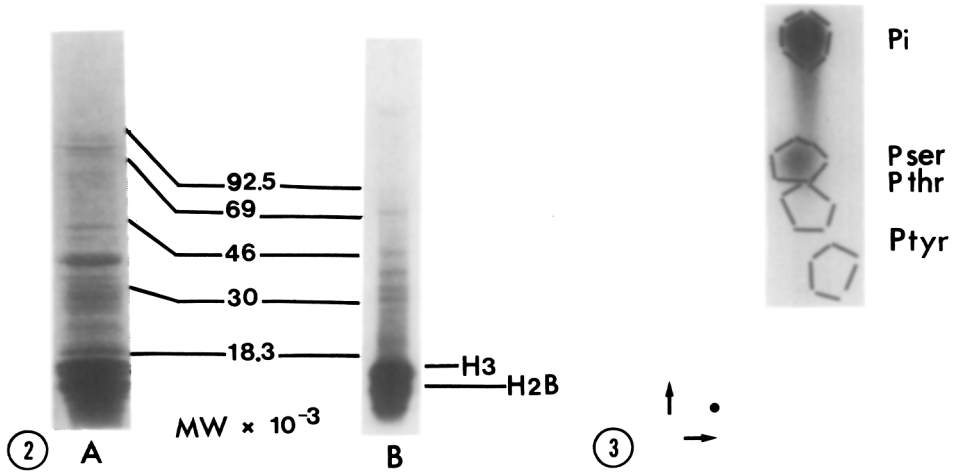

Figure 2. Electrophoretic analysis of DNA bound proteins. DBP phosphorylated *in vitro* in the presence of [γ-^{32}P] ATP were submitted to electrophoresis in SDS-polyacrylamide gel.
Gel A represents a gel stained with Coomassie blue.
Gel B represents the radioautography of a similar gel.

Figure 3. Phosphoaminoacid analysis. DBP were incubated in the presence of [γ-^{32}P] ATP. The incubation medium was extensively dialyzed against water to eliminate free ATP and lyophylized. After acid hydrolysis the aminoacid mixture was resolved in two dimensions on a cellulose thin layer plate by electrophoresis at pH 3.5 for 1 h at 1 kV in acetic acid/pyridine/H_2O (50:5:945) (vertical direction), followed by chromatography in isobutyric acid/0.5 M NH_4OH (5:3) (horizontal direction). The plate was submitted to radioautography.

from ATP (Fig.4). No phosphate transfer occurs in the presence of [γ-^{32}P] GTP.

DISCUSSION

Like most ot the nuclear protein kinases, the enzyme described in this paper is cAMP independent. It has to be compared to the previously described nuclear protein kinases. It differs from protein kinases NI and NII which use casein, phosvitin and the chromosomal non-histone proteins extracted with 1 M NaCl as substrates. NII is able to transfer phosphate from GTP as well as from ATP to serine and also to threonine residues, while the DBP kinase is specific for ATP and transfers the phosphate to serine. NI is unable to phosphorylate any histones (7).

In addition, the DBP kinase is more stable since it was not inactivated by 6 h of incubation at 25°C and can be frozen and defrozen several times without significant loss of activity.

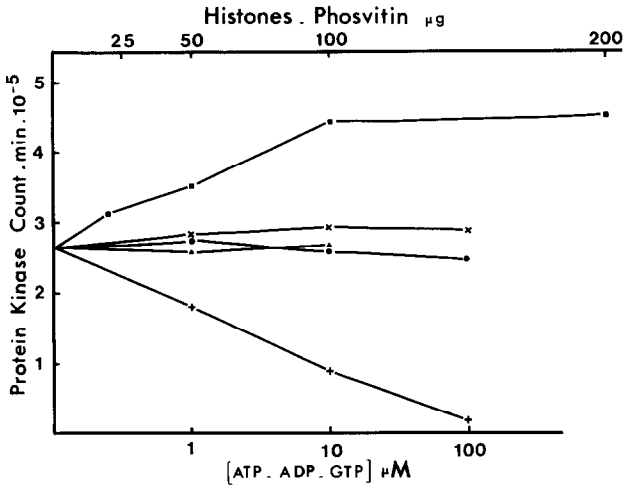

Figure 4. Effect of possible substrates on DBP protein kinase activity. DBP, 100 µg per assay, were incubated at 37°C in the presence of various amounts of total histones(■—■), phosvitin (▲—▲), ATP (+—+), ADP (●—●) and GTP (×—×). The radioactivity of trichloroacetic precipitates was measured on millipore filters.

This enzyme also differs from other nuclear histone kinases : it does not phosphorylate histone H1, it phosphorylates histone H3 on serine instead of threonine residues as does the enzyme described by Shoemaker and Chalkley (8), it is cAMP independent, as opposed to the enzyme described by Taylor (9).

The main characteristics of the DBP-kinase are (a) its strong binding to DNA from which it is not extracted by high ionic strength. It is only solubilized by a complete digestion of DNA (b) its substrate specificity which is restricted to DNA bound proteins : nucleosomal histones, essentially H2B and H3 and a discrete set of non-histone proteins. The specific activity of the enzyme is low, suggesting that it catalyzes the transfer of phosphate on a very limited number of chromosomal proteins. However we cannot exclude that the low specific activity results from a destruction of the chromatin structure, as observed with histone transacetylase (17).

These characteristics strongly suggest that this enzyme could play an important role either on DNA replication or on the control of gene expression. However it is important to ascertain that its specificity *in vivo* is the same as that observed *in vitro* since it has been shown that the specificity

of a chromatin histone kinase (9) and of transacetylases (17,18), is partly lost when the enzymes are incubated with a mixture of substrate proteins instead of nuclei or intact nucleosomes. The specificity of the described enzyme *in vivo* and its biological role are under current investigation.

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