PHOSPHORYLATION OF MOST NON-HISTONE PROTEINS OF CHROMATIN OF RAT LIVER BY AN ATP–GTP-TYPE PROTEIN KINASE

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Received 14 September 1979

1. Introduction

It is well known that numerous non-histone proteins of chromatin are phosphorylated [1]. Protein kinases independent of cyclic nucleotides and which are able to phosphorylate casein and phosvitin but not the histones have been isolated from these proteins. Different authors have reported various numbers of such protein kinases [2–6]. Their physiological substrates are not yet known exactly. Isolated, they phosphorylate the non-histone proteins of chromatin poorly. Here we report that one of the two main protein kinases of casein–phosvitin-type present in chromatin is able to phosphorylate most of the non-histone proteins of chromatin if a thermostable inhibitor has been removed from these proteins. This kinase, which is of high molecular weight, phosphorylates the serine and threonine residues of casein and utilizes GTP as well as ATP.

2. Materials and methods

Phosvitin and casein were obtained from Sigma Labs., [γ-^{32}P]ATP and GTP from the Radiochemical Centre Amersham. Non-histone proteins of chromatin (NHC) were prepared from purified nuclei of male Wag rat liver by saline extractions as in [7]. Protein kinase activity was measured on Whatman disc filters, as in [7,8].

Electrophoretic analysis of phosphorylated NHC: after incubation, proteins were precipitated by trichloroacetic acid (10% final), washed with cold 10% trichloroacetic acid containing 5×10^{-3} M ATP, dissolved in 0.5–1 ml Laemmli sample buffer [9] and dialyzed overnight against 500 ml of the same buffer. Electrophoresis in the presence of SDS was then performed according to [9] using a 10% acrylamide separation gel and was carried out at 110 V for 5 h. After staining with Coomassie blue, gels were dehydrated and dried, and autoradiography was performed by enclosing a flashed Kodak X O MAT R film between the gel and a Dupont lightning screen at −70°C [10] for 1–2 days.

Characterization of phosphorylated amino acids: they were separated after acid hydrolysis of phosphorylated casein on a Dowex column according to [11].

3. Results

Two kinases with phosvitin phosphorylating activities were separated after gel filtration of NHC on a column of Sepharose 6B equilibrated according to [12] for the cytoplasmic enzymes (table 1). The fractions containing the kinases were dialyzed against 0.050 M Tris buffer (pH 7.5), 0.250 M NaCl, 0.002 M $MgCl_2$. Each fraction was then applied to a phosphocellulose column equilibrated with the same buffer and eluted by a discontinuous gradient. The first Sepharose fraction of high molecular weight gives a single peak of phosvitin kinase activity at 0.7 M NaCl. The second fraction of lower molecular weight was eluted at 0.5 M. A minor peak found at 0.7 M may represent a residue of the first activity. Another minor peak was found at 0.4 M. As the cytoplasmic one, the high molecular weight kinase phosphorylates

Table 1
Partial purification of protein kinases I and II of rat liver chromatin

Fraction	Enzymatic activity (UE/mg protein)	Purification	Yield (%)
PNH	0.28	1	100
Sepharose I	2.50	8.90	50
Phosphocellulose I 0.7 M NaCl	18.20	65	20
Sepharose II	1.05	3.75	33
Phosphocellulose II 0.5 M NaCl	12.07	43	21

See text for details

the serine and threonine groups of casein and the second kinase of lower molecular weight only the serine groups (not shown). According to the nomenclature of [13] we designate the first kinase as TS (threonine, serine) and the second one S (serine).

3.1. *Phosphorylation of NHC*

We examined the action of the TS and S enzymes on NHC freed of endogeneous kinase activity either by heating 5 min at 60°C (table 2, line 2), or by passage through a phosphocellulose column (table 2, line 3): the NHC are not phosphorylated by any of the two enzymes. However, if the NHC have been precipitated by ammonium sulfate at 80% saturation, they become phosphorylated by the TS enzyme and to a very small extent by the S enzyme (table 2, lines 2,3).

These results may be attributed to the removal by ammonium sulfate precipitation of an inhibitor. Indeed, NHC precipitated by ammonium sulfate autophosphorylated to a higher degree than if not precipitated (table 2, line 1). Filtration of NHC through a Biogel P10 column also resulted in an increase of autophosphorylation activity, also suggesting the removal of an inhibitor (not shown).

The electrophoretic analysis of the NHC phosphorylated by the TS and S enzymes is shown in fig.1, indicating that TS phosphorylates most of these proteins while S phosphorylates only a few bands. Moreover, direct chromatography of NHC on a phosphocellulose column without previous gel filtra-

Table 2
Phosphorylation of NHC by TS and S enzymes

Incubation medium	^{32}P (nmol incorporated .mg protein^{-1} .min^{-1})		
	–Enzymes TS and S	+ TS Enzyme	+ S Enzyme
Unprecipitated NHC	0.220	0.190	0.200
Precipitated NHC	0.330	0.360	0.320
+ Heating 5 min at 60°C			
Unprecipitated NHC	0.006	0.020	0.007
Precipitated NHC	0.016	0.190	0.050
NHC unadsorbed on phosphocellulose, unprecipitated	0.006	0.017	0.016
NHC unadsorbed on phosphocellulose, precipitated	0.006	0.150	0.050

TS and S enzymes (phosphocellulose step) were tested on NHC, precipitated or not by ammonium sulfate (80% saturation) and devoid of endogeneous kinase activity by heating 5 min at 60°C, or by passage on phosphocellulose column (non-adsorbed fraction) in Tris buffer, 0.050 M Tris, 0.250 M NaCl, 0.002 M $MgCl_2$ at pH 7.5. Incorporation was measured after 10 min incubation at 37°C with 10^{-4} M [γ-^{32}P]ATP, 1 mg protein/ml, 30 μg enzyme

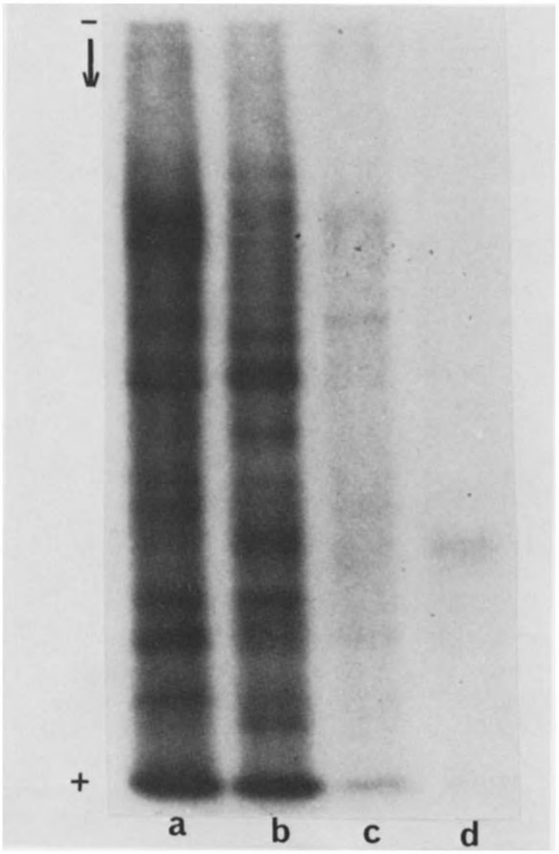

Fig.1. Phosphorylation of NHC by TS and S enzyme. Autoradiographic analysis. NHC devoided of endogeneous kinase activity or by heating 5 min at 60°C or by passage through a phosphocellulose column, precipitated by ammonium sulfate 80% saturation have been incubated 10 min at 37°C with 10^{-4} M [γ-^{32}P]ATP and TS and S enzyme. After arrest of the reaction with Laemmli sample buffer, the samples were electrophorezed and autoradiographed as indicated in section 2. Track (a) heated NHC + TS enzyme; track (b) unadsorbed NHC + TS enzyme; track (c) heated NHC + S enzyme; track (d) unadsorbed NHC + S enzyme.

tion shows that only the peak eluted at 0.7 M NaCl corresponding to the position of the TS enzyme phosphorylates the NHC (fig.2).

Further evidence that the TS enzyme is responsible for the phosphorylation of most NHC is that autophosphorylation of NHC may be carried out with GTP as well as ATP (though with less efficacity) as the TS enzyme while S enzyme utilized only ATP (table 3).

4. Discussion

We have shown that among the two main phosvitin–casein kinases of chromatin of normal rat liver, one is able to phosphorylate most of the non-histone proteins of chromatin (if an inhibitor has been removed). This kinase of relatively high molecular weight (~150 000, unpublished results), which may be analogous to the kinase purified from whole nuclei of rat liver [14], phosphorylates the serine and threonine groups of casein and utilizes GTP as well as ATP. However, it is not excluded that these fractions could represent a family of enzymes, each specifically phosphorylating a small number of NHC. The small peak found in phosphocellulose at 0.4 M NaCl may be responsible for the phosphorylation of some NHC, as suggested [15]. The S enzyme phosphorylates poorly and only a limited number of these proteins. One enzyme from yeast and Novikoff ascites tumor with properties analogous to the S enzyme has been copurified with the RNA polymerase and is able to phosphorylate some of its subunits [16–18].

Our results suggest the presence in chromatin of an inhibitor of protein kinase TS. This inhibitory fraction is thermostable, as shown by the inability of the TS enzyme to phosphorylate the heated NHC if the inhibitor has not been removed by ammonium sulfate precipitation of NHC. It is a macromolecule, as it is non-dialysable through membranes with an exclusion limit of 10 000 but its behaviour on gel filtration and its solubility in 80% ammonium sulfate suggest a small molecular weight. A specific thermo-

Table 3
Specificity toward nucleotide triphosphates

Substrates	^{32}P (nmol incorporated .mg protein^{-1} .min^{-1})	
	+ [γ-^{32}P]ATP	+ [γ-^{32}P]GTP
NHC	0.26	0.20
Phosvitin + TS enzyme	1.20	0.70
Phosvitin + S enzyme	0.90	0.01

Incorporation was measured after 10 min incubation with [γ-^{32}P]ATP or [γ-^{32}P]GTP 10^{-4} M, 1 mg proteic substrate/ml and 30 μg purified enzyme (phosphocellulose step)

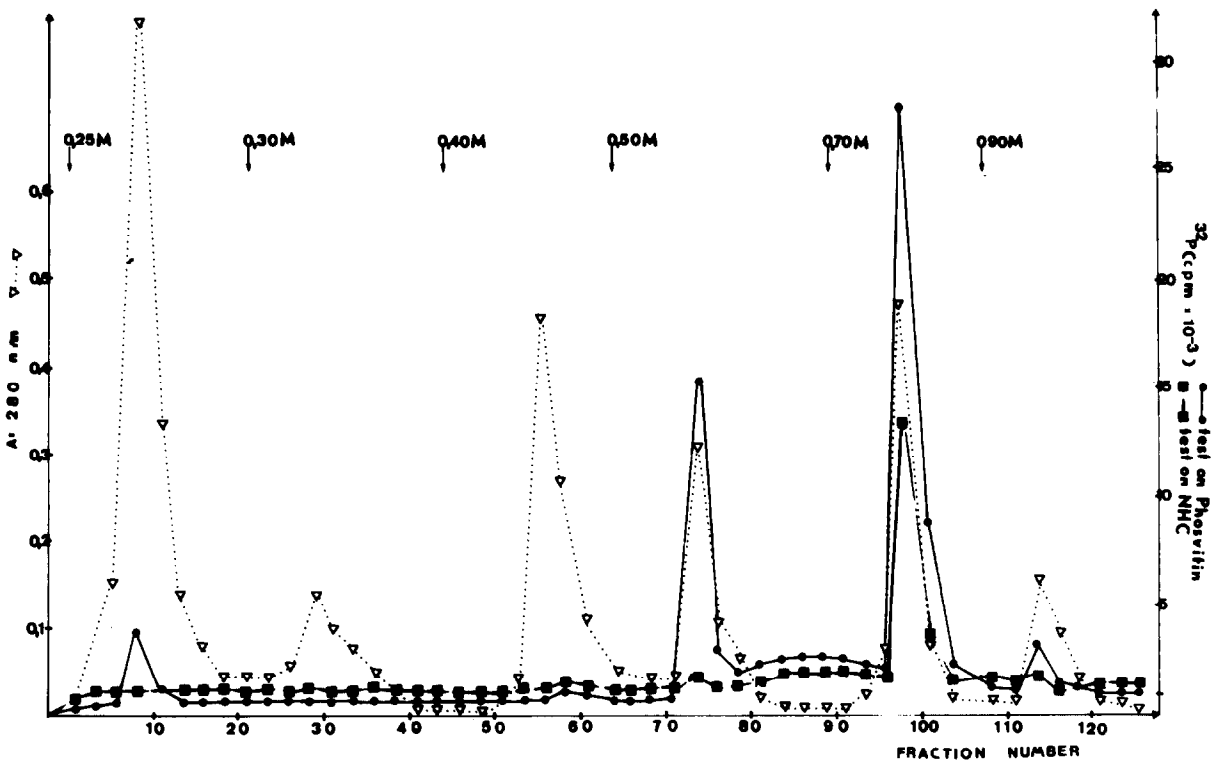

Fig.2. Chromatography of NHC on phosphocellulose. NHC are applied on a phosphocellulose column (17 X 2.5 cm) equilibrated with buffer. 0.050 M Tris 0.250 M NaCl, 0.002 M $MgCl_2$. They were eluted by a discontinuous gradient of NaCl in 0.050 M Tris, 0.002 M $MgCl_2$ at pH 7.5; 3 ml fractions were collected and kinase activity tested on phosvitin and NHC precipitated by ammonium sulfate at 80% and heated 5 min at 60°C.

stable polypeptide inhibitor of the cyclic AMP-dependent protein kinases has been known for some years and is well characterized [19,20]. But until now no specific inhibitor has been found for the cyclic nucleotide-independent kinases. However, while our work was in completion, a polypeptide inhibiting a casein–phosvitin kinase was reported [21] in surrenal cytoplasm. The inhibitor presented here may be analogous. The inhibition of phosvitin–casein kinase by nucleic acids (especially the tRNA) has also been reported [22]. Further work is necessary to clarify the nature of the inhibitor.

Acknowledgements

This work was financially supported by the Centre National de la Recherche Scientifique and la Délégation Générale à la Recherche Scientifique et Technique.

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