STUDIES ON THE PHOSPHORYLATION OF NUCLEOLAR PROTEINS

Identification of a nucleolus-associated protein kinase activity

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1. Introduction

In earlier studies it could be shown that most agents which stimulate RNA synthesis in animal cells affect initially the synthesis of pre-rRNA in the nucleolus [1, 2]. The rates of phosphorylation of nuclear proteins are increased in cells undergoing such gene activation [3–7]. Whether, however, the regulation of tRNA synthesis is accompanied by reversible phosphorylation of specific nucleolar proteins is a still unresolved problem.

This paper describes a protein kinase activity which is associated with highly purified nucleoli. This enzyme catalyzes the phosphorylation of both saline-soluble and chromosomal nucleolar proteins.

2. Materials and methods

[γ-^{32}P] ATP was prepared according to Gibbs et al. [8] or was obtained from the Radiochemical Centre (Amersham). Rabbit skeletal muscle protein kinase was prepared by the method of Walsh et al. [9]. 3′5′ cAMP and ADP were pruchased from Boehringer (Mannheim), Triton X-100 and dithiothreitol were from Serva (Heidelberg).

Nucleoli were isolated from rat liver as described previously [10]. The purified nucleoli were free of contaminating nuclei or extranucleolar chromatin as checked by phase contrast and electron microscopy. They could be stored in liquid nitrogen without loss of protein kinase activity.

For the assay of protein kinase activity the nucleoli (20–40 μg protein) were incubated at 30°C in a total volume of 0.1 ml in 250 mM sucrose; 100 mM Tris–HCl, pH 7.5; 20 mM $MgCl_2$; 100 mM NaCl; 6 mM NaF; 10 mM dithiothreitol and 0.005 mM [γ-^{32}P]-ATP (0.5–1.2 Ci/mmole). The reaction was terminated by cooling the tubes and then adding 0.5 ml of 5% trichloroacetic acid. After hydrolysis of nucleic acids by heating to 90°C for 20 min the acid-insoluble material was collected on glass fibre filters (Whatman GF/A), washed with cold 5% trichloroacetic acid and assayed for radioactivity.

3. Results and discussion

If rat liver nucleoli were incubated in vitro with [γ-^{32}P] ATP a considerable incorporation of radioactivity into acid-precipitable material occurs. On the average more than 80% of the acid-insoluble counts were incorporated into protein which agrees with the results of others [11, 12]. Less than 2% of the total insoluble counts were incorporated into lipid material. The amounts of phosphate incorporated in the presence of 0.5 nmole [γ-^{32}P] ATP was proportional to the amount of nucleoli between 3 μg and at least 30 μg nucleolar protein. Within this range approx.

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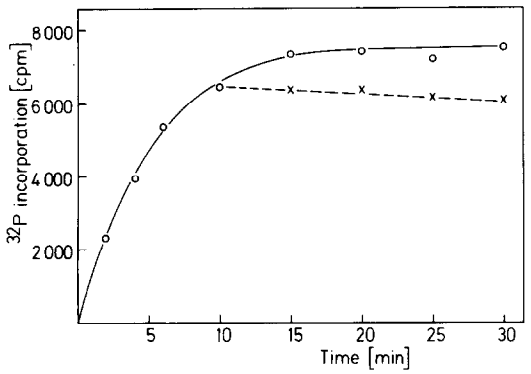

Fig. 1. Time course of in vitro phosphorylation of nucleolar proteins. The nucleoli were incubated at 0.3 mg protein/ml as described in Materials and methods and 0.1 ml samples were periodically withdrawn at the times indicated. After 10 min the reaction mixture was divided, an excess of inactive ATP was added to one part of the mixture and the incubation continued. (o—o—o) incubation with 5 mμmoles of [γ-^{32}P] ATP; (x--x--x) addition of an excess of inactive ATP (50 mμmoles/ml) after 10 min.

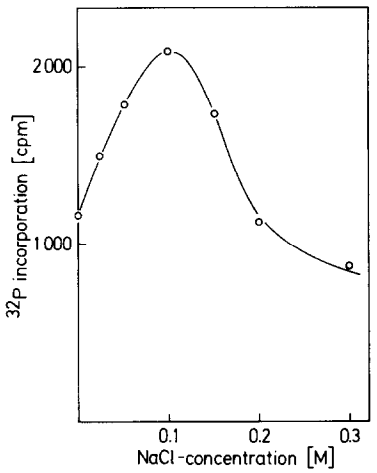

Fig. 2. Effect of NaCl on the phosphorylation reaction in isolated nucleoli. Incubation conditions were as described in Materials and methods; incubation time was 5 min.

0.1 pmole phosphate per μg protein was incorporated in 5 min at 30°C.

The time course (fig. 1) of the incorporation of ^{32}P into the protein fraction showed that the phosphorylation reaction was essentially completed after 15 min incubation. When the radioactive ATP was diluted by a 10-fold excess of unlabeled ATP after 10 min incubation, no marked decrease in total radioactivity was observed. This indicates a very low turnover of phosphate groups under the conditions used.

The phosphorylation reaction is considerably stimulated by monovalent ions. Fig. 2 shows the influence of increasing amounts of NaCl. Maximum incorporation of ^{32}P occurs at 0.1 M NaCl, whereas concentrations higher than 0.2 M NaCl inhibit the reaction.

In another set of experiments the influence of 3′5′ cyclic AMP, skeletal muscle protein kinase as well as exogenous substrate on the phosphorylation reaction was investigated.

It is well known that skeletal muscle protein kinase responds in activity to 3′5′ cyclic AMP whereas the chromatin associated protein kinase activity is not stimulated [13, 14]. In table 1 it is shown that the nucleolus-associated protein kinase activity is not affected by cyclic AMP. Similarly, the addition of skeletal muscle protein kinase did not stimulate the phosphorylation of nucleolar proteins.

If, however, ribosomal proteins from rat liver are added a considerable enhancement of the phosphorylation reaction was observed. This indicates that the enzyme(s) responsible for the phosphorylation of nucleolar proteins are in excess over their substrate.

In the accompanying paper [15] it is shown that the phosphorylation capacity (pmoles $^{32}PO_4$ incorporated per μg protein) of isolated nucleoli is several times higher than that of isolated nuclei. This fact strongly supports the idea that the nucleolus-associated protein kinase activity is not unspecifically adsorbed to these organelles but represents an integral part of the nucleolus.

The K_M values of the two chromatin bound protein kinases for ATP were reported to be 1.1×10^{-5} M and 2.0×10^{-5} M [13]. In our system an estimate of the affinity of the nucleolar enzyme(s) for ATP was obtained by incubating a constant amount of nucleoli with increasing amounts of [γ-^{32}P] ATP. The apparent K_M value for ATP is 2×10^{-6} M calculated by the use of the Lineweaver and Burk plot. ADP, which competitively inhibits the protein kinase(s) lowers the K_M value to 1.8×10^{-5} M ATP if present in a 20-fold excess over ATP (fig. 3). The estimated K_M value of the nucleolar protein kinase(s) for ATP is thus an order of magnitude lower than

Table 1
Effect of 3'5' cAMP, skeletal muscle protein kinase and ribosomal proteins on the phosphorylating activity of isolated nucleoli.

System	Radioactivity incorporated (cpm)	%
Nucleoli (alone)	3410	100%
Nucleoli + cAMP	3300	97%
Nucleoli + cAMP + protein kinase	3240	95%
Nucleoli + ribosomal proteins	7980	234%

The reaction mixture contained 30 μg nucleolar protein. Where indicated 10^{-5} M cAMP or 30 μg ribosomal proteins were added. Incubation was for 15 min at 30°C.

those of the nuclear chromatin-associated enzymes. But isolation and purification of the nucleolar enzyme(s) will be necessary to determine whether or not these enzymes are different.

Analysis of the in vitro phosphorylated proteins by gel electrophoresis of saline-soluble and chromosomal nucleolar proteins revealed a distribution of proteins phosphorylated in vitro similar to those phosphorylated in vivo [15]. The bulk of ^{32}P activity incorporated was found in the non-histone chromatin proteins. The phosphorylation of non-histone proteins had been intimately related to chromosome structure and gene activation [3–6]. At present it is not known whether the extent of phosphorylation of nucleolar proteins influence the rate of rRNA synthesis. However, it is now possible to investigate whether there is any correlation between phosphorylation and rRNA synthesis in isolated nucleoli.

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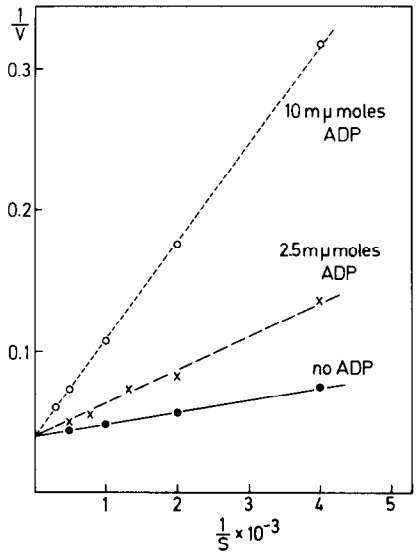

Fig. 3. Amount of phosphate incorporated in a 5 min incubation period as a function of the [γ-^{32}P] ATP concentration in the presence and absence of ADP. Reciprocals of the substrate concentration in pmoles ($1/S$) plotted against the reciprocals of the phosphorylation reaction expressed as pmoles $^{32}PO_4$ incorporated ($1/v$) into protein. The reaction mixture contained 5 mμmoles/ml [γ-^{32}P] ATP and 100 μg/ml nucleolar protein. (o – o – o) incubation mixture without ADP; (x – – x – – x) 2.5 mμmoles/ml ADP in the assay; (o – – o – – o) 10 mμmoles/ml ADP in the assay.

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