PHOSPHORYLATION OF A NUCLEOLUS-SPECIFIC PHOSPHOPROTEIN *IN VITRO*

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SUMMARY

The cellular site and characteristics of the phosphorylation of a nucleolus-specific phosphoprotein (molecular weight, 120 000) in mouse ascites tumor cells were studied. The phosphoprotein was strongly labeled with ^{32}P when the isolated nucleoli were incubated with [γ-^{32}P]ATP *in vitro*. This phosphoprotein, and protein kinase for the protein phosphorylation were both purified from 0.3 M KCl soluble protein fraction of the nucleoli by hydroxylapatite and phosphocellulose column chromatographies. It was found that phosphorylation of the nucleolus-specific phosphoprotein was catalyzed selectively by a guanosine 3:5-monophosphate-dependent protein kinase in the nucleoli and the reaction product was the same phosphoprotein as the substrate used.

Previously, we reported the existence of a nucleolus-specific phosphoprotein in mouse ascites tumor cells with a molecular weight of 120 000 in which the phosphate moiety turned over rapidly (1). We also found that this unique phosphoprotein was essential for preservation of the stereo-specific structure of the nucleoli. Thus analyses of the site and mechanism of phosphorylation of this phosphoprotein seemed important for elucidating the biological function of this protein in regulation of the nucleolar structure. There are reports on the phosphorylation of nucleolar proteins *in vitro* (2-4), but the mechanism of the phosphorylation has not yet been analyzed using purified specific nucleolar proteins.

This paper deals with characterization of the phosphorylation of the nucleolus-specific phosphoprotein catalyzed by a guanosine 3:5-monophosphate-(cyclic GMP)-dependent protein kinase from the nucleoli.

MATERIALS AND METHODS

The method used for isolation of nucleoli from mouse ascites tumor cells 4 days after transplantation was described elsewhere (5,6). [γ-^{32}P]ATP, ammonium salt (15-17 Ci/mmole) was a product of the Radiochemical Centre, Amersham. Cyclic GMP, Na-salt and cyclic AMP, Na-salt were purchased from Sigma Chemical Co., St. Louis, Mo. Hydroxylapatite, Bio-Gel HTP was obtained from Bio-Rad Laboratories, Richmond, Calif. Phosphocellulose, Whatman P-11 was a product of W. and R. Balston, Maidstone, Kent.

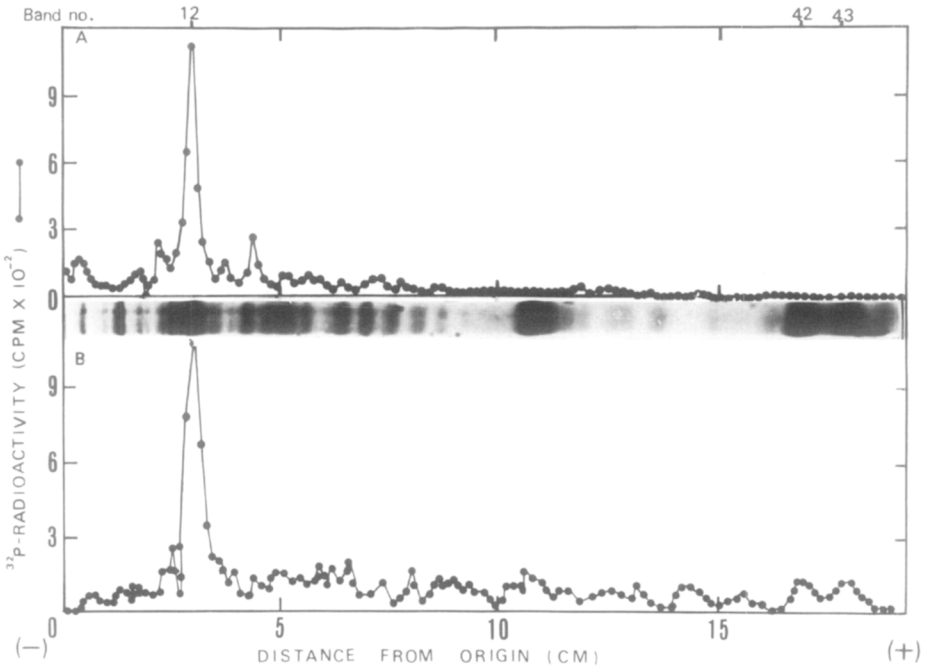

Figure 1. SDS-polyacrylamide gel electrophoresis of nucleolar proteins after phosphorylation *in vitro* and *in vivo*. A) Proteins in the isolated nucleoli labeled with [γ-^{32}P]ATP for 20 min *in vitro*; B) proteins in the nucleoli from the tumor cells labeled with [^{32}P]orthophosphate for 1 h *in vivo* (1). (●——●) radioactivity, counts/min/mm slice.

Phosphorylation of proteins in isolated nucleoli in vitro. Isolated nucleoli were incubated in a reaction mixture (0.5 ml) containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$ and 25 µCi [γ-^{32}P]ATP at 37° for 20 min. The reaction was ceased by adding a final 1 percent SDS. The proteins prepared as described previously (1) were subjected to SDS-polyacrylamide gel electrophoresis (7). The protein bands in the gel were analyzed with Coomassie brilliant blue staining and distribution of ^{32}P radioactivity among them.

Isolation of the nucleolus-specific phosphoprotein and protein kinase. Purification of the nucleolus-specific phosphoprotein by hydroxylapatite column chromatography without SDS was based on the procedures in the previous paper (1). For preparation of protein kinase the unadsorbed fraction from the column was applied to a phosphocellulose column. The column was eluted with a linear gradient of 0.2 to 0.8 M NaCl in 50 mM Tris-HCl (pH 7.5) at 4° (8). To examine the distribution of protein kinase activity in the eluate, 0.1 ml aliquot of each fraction was mixed with a solution (1 ml) containing 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 10 mM $MgCl_2$, 0.1 mg bovine casein as substrate and 1 µCi [γ-^{32}P]ATP, and incubated for 20 min at 37°. The enzyme activity was assayed by measuring the radioactivity in the hot trichloroacetic acid insoluble fraction.

Phosphorylation of the phosphoprotein by protein kinase in vitro. Phosphorylation of the nucleolus-specific phosphoprotein *in vitro* using a protein kinase fraction eluted with 0.55 M NaCl from a phosphocellulose column was

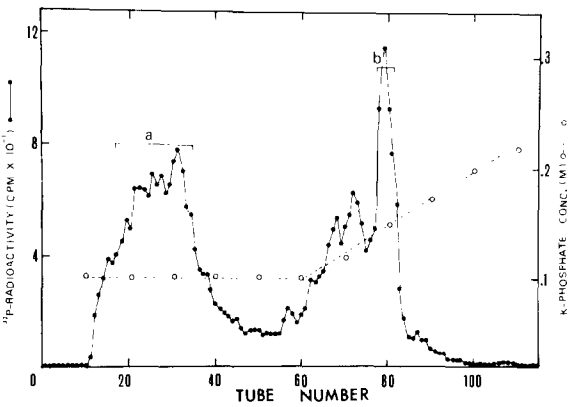

Figure 2. Chromatogram of 0.3 M KCl soluble nucleolar proteins on a hydroxylapatite column without SDS. The nucleolar proteins solubilized in 0.3 M KCl solution were prepared from tumor cells labeled with [^{32}P]orthophosphate for 1 h *in vivo*. The frames in this figure indicate the unadsorbed fraction used for further purification of protein kinases (a) and the protein fraction used for the nucleolus-specific phosphoprotein (b). (●---●) radioactivity, counts/min/0.1 ml fraction; (○····○) potassium phosphate concentration, M.

examined using the same reaction mixture (1 ml) as that described in the preceding section plus 10^{-5} M cyclic GMP for 20 min at 37°. Incorporation of ^{32}P from [γ-^{32}P]ATP into the phosphoprotein was analyzed by determination of radioactivity in the hot acid insoluble fraction and by SDS-polyacrylamide gel electrophoresis as described above.

RESULTS AND DISCUSSION

It has been previously demonstrated that a phosphoprotein with a molecular weight of 120 000 is localized in the nucleoli of mouse ascites tumor cells (1). In this work we examined whether this phosphoprotein could be phosphorylated in the nucleoli. Figure 1 shows the profiles of incorporation of ^{32}P from [γ-^{32}P]ATP *in vitro* (Figure 1A) and from [^{32}P]orthophosphate *in vivo* (Figure 1B) (1) into nucleolar proteins on SDS-polyacrylamide gel electrophoresis. The profiles were similar because the nucleolus-specific phosphoprotein, band no. 12 was strongly labeled and most other protein bands contained some ^{32}P under these different conditions. This shows that this nucleolus-specific phosphoprotein was phosphorylated in the nucleoli *in situ*. One distinct difference between the profiles of radioactivity was that histone proteins, bands no. 42 and 43 were labeled appreciably *in vivo*, but not *in vitro*. This suggests that histone proteins of the nucleoli may be phosphorylated outside of this organelle.

Since these results indicated that the nucleolus-specific phosphoprotein was phosphorylated in the nucleoli, we next attempted to isolate the phospho-

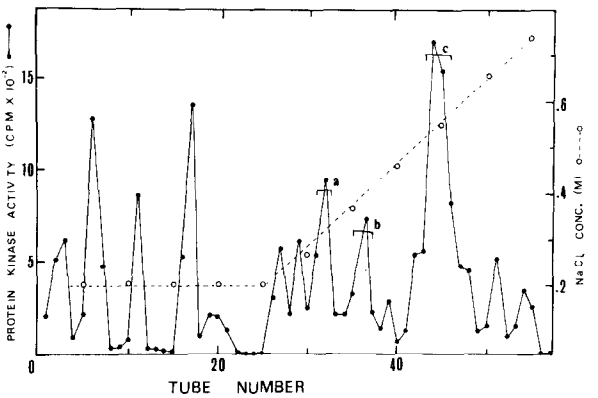

Figure 3. Chromatogram of protein kinase fractions on a phosphocellulose column. The unadsorbed fraction from the hydroxylapatite column (frame a in Figure 2) was applied to a phosphocellulose column. Protein kinase activity in the eluate was determined as described in Materials and Methods. The frames in this figure indicate protein kinase fractions used as enzyme source, a) 0.32 M NaCl, b) 0.40 M NaCl and c) 0.55 M NaCl fractions. (●——●) protein kinase activity, counts/min/0.1 ml fraction; (○···○) NaCl concentration, M.

protein and protein kinase from the nucleoli for further analysis on properties of the reaction. As demonstrated in the previous work, the phosphoprotein was extracted from the nucleoli with 0.3 M KCl and isolated as a single component by hydroxylapatite column chromatography without SDS (1). The protein fraction eluted from the column with 0.14 M potassium phosphate buffer consisted entirely of the phosphoprotein (Figure 2 and ref. 1). It was tested that the phosphoprotein in this fraction had no protein kinase activity.

Next, purification of protein kinase from 0.3 M KCl soluble protein fraction was carried out, because of more than 90 percent of the enzyme activity of the nucleoli was also found in this fraction. For this purpose, the unadsorbed fraction (frame a in Figure 2) was charged on to a phosphocellulose column and the column was eluted with a linear gradient of 0.2 to 0.8 M NaCl in 50 mM Tris-HCl (pH 7.5) (8). Protein kinase activity in the eluate was assayed by measuring ^{32}P incorporation from [γ-^{32}P]ATP into the substrate, bovine casein *in vitro*. As shown in Figure 3 multiple fractions of protein kinase were found in the eluate and the highest activity was reproducibly detected in a fraction eluted with 0.55 M NaCl. There are reports of multiple protein kinase in nuclear and chromatin proteins (8-10), but not in nucleoli (2-4).

In relation to this subject we tested their enzyme and substrate specificities using different fractions with activity from the column eluate

Table 1. Substrate and enzyme specificities of the phosphorylation *in vitro*

Substrate	Protein kinase fraction	Protein kinase activity	
		counts/min x 10^{-4} per mg protein	Relative activity
	Expt. 1		
Nucleolus-specific phosphoprotein	0.55 M NaCl (c)	16.5	100
	0.40 M NaCl (b)	5.2	31
	0.32 M NaCl (a)	0.4	2
	Expt. 2		
Nucleolus-specific phosphoprotein		75.7	100
Casein		1.6	2
Histone	0.55 M NaCl (c)	0.2	0.3
Phosvitin		0.2	0.3
Protamine		0.02	0.03

The incorporations of ^{32}P from [γ-^{32}P]ATP into various protein substrates catalyzed by the protein kinase fractions were analyzed under standard assay conditions as described in Materials and Methods. The protein kinase fractions used are shown by frames a, b and c in Figure 3.

and various protein substrates. The enzyme in the unadsorbed fraction from the phosphocellulose column was not tested because it was contaminated with other phosphoproteins. Table 1 shows that the fraction eluted with 0.55 M NaCl had high activity for phosphorylation of the nucleolus-specific phosphoprotein, but that the kinases eluted with 0.32 M and 0.40 M NaCl did not catalyze this phosphorylation appreciably. On the other hand, the protein kinase eluted with 0.55 M NaCl showed much higher activity with the phosphoprotein than with commercially available proteins, casein, histone, phosvitin and protamine. Thus protein phosphorylation in the nucleoli seems to show both enzyme and substrate specificities. This is consisted with results on protein kinases prepared from nuclear and chromatin proteins (8-10). The exact specificities of these protein kinases in the nucleoli require investigation using different protein fractions isolated from the nucleoli.

The phosphorylation showed the following characteristics: 1) Phosphorylation continued linearly for at least 20 min (Figure 4A) and was proportional to the amount of substrate added (Figure 4B). 2) Mg^{2+} was essential for activity, and its optimal concentration was 10 mM. Other divalent cations, Ca^{2+} and Zn^{2+} also stimulated the reaction, but their

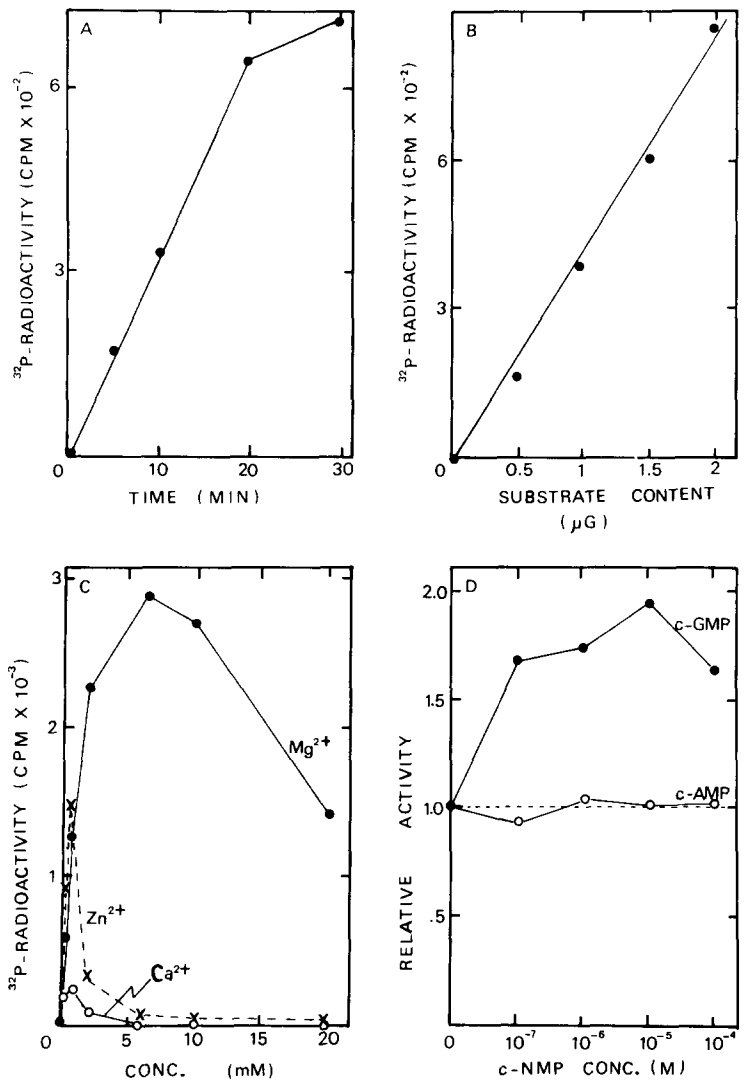

Figure 4. Various properties of the phosphorylating reaction of the nucleolus-specific phosphoprotein *in vitro*. The reaction was carried out under the standard conditions described in Materials and Methods. A) Time course of the reaction; B) Effect of substrate content; C) Effects of divalent cations; D) Effects of cyclic mononucleotides.

effects were less than that of Mg^{2+} at their optimum concentrations of 0.2 and 0.6 mM, respectively (Figure 4C). 3) Na^{+} was also required for maximal activity and its optimal concentration was around 0.2 M. 4) The optimum pH of the reaction was 7.5. 5) When cold 5 mM ATP was added to the reaction mixture after the labeling with [γ-^{32}P]ATP for 20 min, 57 percent of the ^{32}P radioactivity was chased out. An interesting fact to be noted here was effect of cyclic mononucleotides on phosphorylation of the nucleolus-specific

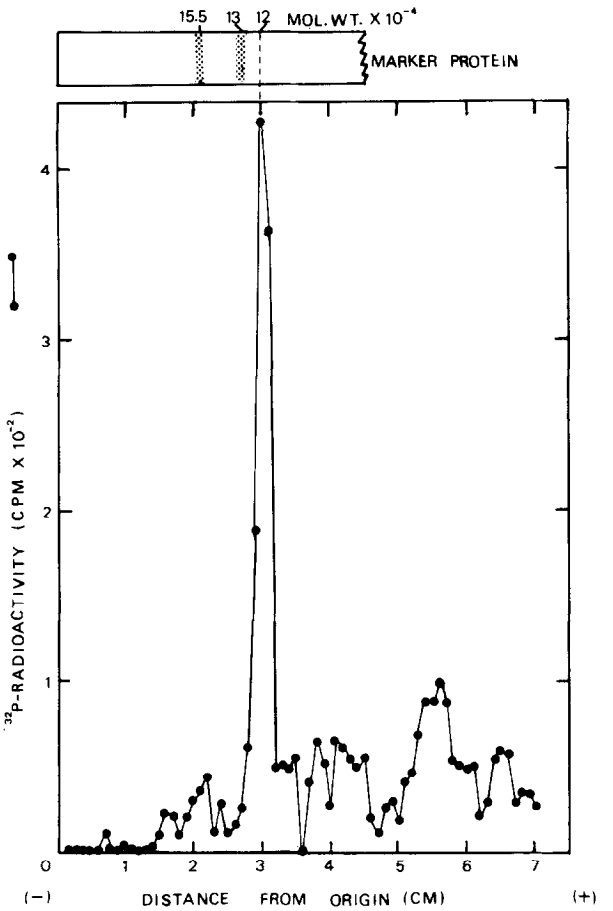

Figure 5. Analysis of the reaction product of phosphorylation of the nucleolus-specific phosphoprotein *in vitro* by SDS-polyacrylamide gel electrophoresis. The procedures for both incubation with [γ-^{32}P]ATP *in vitro* and analysis of ^{32}P labeled phosphoprotein were described in Materials and Methods. RNA polymerase, β subunit (mol. wt. 155 000) and β-galactosidase (mol. wt. 130 000) of *E. coli* were used as marker proteins for molecular weight determination. (●– –●) radioactivity, counts/min/mm gel slice.

phosphoprotein *in vitro*. Cyclic GMP at concentrations of 10^{-7} to 10^{-4} M stimulated the phosphorylation 50 to 100 percent, whereas cyclic AMP did not (Figure 4D). There are no previous reports that cyclic mononucleotides affect the phosphorylation of nucleolar proteins *in vitro* (2-4).

To confirm that the nucleolus-specific phosphoprotein with a molecular weight of 120 000 was actually phosphorylated by the cyclic GMP-dependent protein kinase from the nucleoli, we analyzed reaction product by SDS-polyacrylamide gel electrophoresis. Figure 5 shows that ^{32}P radioactivity locates exclusively on the nucleolus-specific phosphoprotein with a molecular

weight of 120 000. When the reaction product was hydrolyzed with pronase (10 μg/mg), alkaline phosphatase (20 μg/mg) and hot 1 N NaOH, 99, 55 and 100 percent, respectively of the ^{32}P was recovered in the acid soluble fraction. But treatment with 5 percent trichloroacetic acid at 90° for 20 min which hydrolyzes both RNA and DNA completely, did not liberate radioactivity from the phosphoprotein.

From these experimental results it can be concluded that this unique phosphoprotein localized in the nucleoli is phosphorylated in the nucleoli by a cyclic GMP-dependent protein kinase. As reported previously, the phosphate moiety of this phosphoprotein has a very high turn-over rate (1). Therefore, both active phosphorylation of this phosphoprotein by the protein kinase and dephosphorylation by a phosphoprotein phosphatase must be important in regulation of the structure and function of the nucleoli. It was also demonstrated previously that the divalent cations, Ca^{2+} and/or Mg^{2+} were found to be essential for preservation of nucleolar structure (1). The present work showed that they were required for activity of protein kinase on the phosphorylation of the unique phosphoprotein. Thus their effect on nucleolar structure may be related to their actions on the phosphoprotein and/or the enzymes concerned with its phosphate metabolism. Attempts to determine whether the phosphoprotein interacts with these divalent cations *in vitro* are in progress.

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