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RESOLUTION AND GENERAL PROPERTIES OF DIFFERENT TYPES OF RIBOSOMAL PROTEIN KINASES IN MOUSE PLASMOCYTOMA

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Summary

Three different types of protein kinases (ATP : protein phosphotransferase, EC 2.7.1.37) were isolated and partially purified from a mouse plasmacytoma microsomal KCl wash fraction, then chromatographed on DEAE cellulose and phosphocellulose. The three protein kinase activities designated by protein kinase I, II and III were characterized with respect to their capacity to utilize [γ-^{32}P]ATP and [γ-^{32}P]GTP, to interact with cyclic AMP, stimulation by cyclic AMP, substrate specificity and sedimentation behaviour on glycerol gradient centrifugation.

Protein kinase I was found to be cyclic AMP dependent and preferentially phosphorylated histones. Protein kinase II and III were insensitive to cyclic AMP, protein kinase II preferentially phosphorylated histones and the protein(s) of a ribosomal KCl wash fraction eluted from DEAE cellulose between 0.2 and 0.35 M KCl and termed "PPx". Protein kinase III phosphorylated casein and ribosomal proteins to a great extent. Studies with glycerol density gradient centrifugation indicated that protein kinase I sediments as a component of about 4.4 S, protein kinase II of 4.3 S and protein kinase III of 3 S.

Chromatography on phosphocellulose of the protein kinases isolated from purified free polysomes showed the same type of protein kinases as those from microsomes. So it appears unlikely that protein kinase I and II were contaminants from the cytosol.

Introduction

Protein kinases (ATP : protein phosphotransferases, EC 2.7.1.37) have been described in various mammalian cells [1] in yeast [2] and in plant cells [3]. In mammalian cells, they are widely distributed in the different subcellular fractions; they are localized in the cytosol [4—7] on ribosomes [2,3,8,9], in the

nucleus [10—13] on plasma membranes [14,15] and on free messenger ribonucleoprotein particles [24]. They catalyse the transfer of the γ-phosphate of ATP or GTP to nuclear proteins [10—13], ribosomal proteins [4,6,7,16,17] or plasma membrane proteins [14,15].

Some of these enzymes have been found to be activated by cyclic AMP while others were cyclic AMP independent [1]. This communication describes the partial purification and the main characteristics of three different types of protein kinases associated with the microsomes in mouse plasmacytoma.

Material and Methods

$^{32}PO_4$ was purchased from CEA (Gif-sur-Yvette, France), [γ-^{32}P]ATP and [γ-^{32}P]GTP was from New England Nuclear Corporation (specific activity 14—23 Ci/mmol), cyclic AMP was obtained from Boehringer (Mannheim, G.F.R.), whole histone from calf thymus was a gift from Dr. Gross-Bellard, casein was bought from Difco Whatman DE. 32. DEAE cellulose and P11 phosphocellulose from W. and R. Baldstone (Maidstone, U.K.).

Preparation of microsomal KCl wash

RPC_5 plasma cell tumors, transplanted in Balb/c mice were used. Tumors were homogenized in 2.5 vol. of buffer A (0.03 M Tris · HCl, pH 7.6; 25 mM KCl; 5 mM magnesium acetate; 1 mM dithiothreitol; 0.8 M sucrose) and centrifuged at 20 000 × *g* for 10 min. The pellet containing microsomes and free ribosomes was obtained from the supernatant by sedimentaion at 160 000 × *g* for 90 min.

The particulate fraction was suspended in buffer B (35 mM Tris; 1 mM dithiothreitol; 0.25 M sucrose) to give 250 A_{260} units/ml then brought to 0.6 M with respect to KCl. After slowly stirring in the cold for 30 min, the suspension was centrifuged at 250 000 × *g* for 90 min. The upper three-quarters of the supernatant were removed. This fraction is called the "KCl wash".

DEAE cellulose chromatography

The KCl wash was brought to 70% saturation with ammonium sulphate. The precipitate was dissolved in buffer C (10 mM Tris · HCl pH 7.6; 0.2 mM EDTA; 1 mM dithiothreitol; 10% glycerol) containing 0.12 M KCl and dialyzed against this buffer for 15 h.

Proteins were applied to 1.5 × 30 cm column of DEAE cellulose equilibrated with buffer C containing 0.05 M KCl and eluted with a 0.05 M to 0.5 M linear gradient of KCl in buffer C. 3 ml fractions were collected and 50 μl samples were used for the protein kinase assay.

Phosphocellulose chromatography

(a) Protein kinases. The major peak of protein kinase activity obtained after DEAE cellulose chromatography was brought to 50% saturation with ammonium sulphate. The precipitate was dissolved in buffer C containing 0.12 M KCl and dialyzed against this buffer for 15 h. Proteins were applied to a 1 × 19 cm column of P cellulose equilibrated with buffer D (10 mM Tris · HCl, pH 7.9; 0.2 mM EDTA; 1 mM dithiothreitol; 5% glycerol) containing 50 mM KCl and

eluted with a 0.05 M to 0.7 M linear gradient of KCl in buffer D. 3-ml fractions were collected and 50 μl samples served for protein kinase assay. Each peak of protein kinase activity was concentrated by Diaflo ultrafiltration (UM 10) and stored under liquid nitrogen.

(b) Protein(s) from "PPx" fraction. The ^{32}P-labelled plasmacytoma ribosomal KCl wash was prepared from mice injected intraperitoneally with 1.5 mCi of $^{32}PO_4$ and killed after 1 h. The ^{32}P-labelled fraction eluted from DEAE cellulose between 0.2 and 0.35 M KCl as we have shown in a previous paper [18] was called PPx. This fraction was brought to 70% saturation with ammonium sulphate. The precipitate was dissolved in buffer E (10 mM Tris · HCl, pH 7.9; 0.2 mM EDTA; 1 mM dithiothreitol; 1 M urea) containing 0.1 M KCl and dialized against this buffer for 15 h. 1—2 mg of proteins were applied to a 1 × 12 cm column of P cellulose equilibrated with buffer E and eluted with 0.1—0.5 M stepwise gradient of KCl in buffer E. A sample of 1 ml of each column fraction was removed and added to 1.5 ml of distilled water. The sample was then mixed with 5 ml Instagel (Packard) with vigorous shaking. The radioactivity was measured by liquid scintillation spectrometry (Intertechnique ABAC/SL 40). Either ^{32}P-labelled or nonlabelled peak of "PPx" was concentrated by Diaflo ultrafiltration (UM 10) and stored under liquid nitrogen.

Protein kinase assay

The incubation mixture contained: 35 mM Tris · HCl, (pH 7.6); 5 mM magnesium acetate; 2 mM dithiothreitol; 15 nmol [γ-^{32}P]ATP (specific activity 4—6 · 10^4 cpm/nmol; 40—80 μg protein substrate; 50 μl of the column fractions or 10—20 μg partially purified protein kinase and cyclic AMP when indicated. The samples in a total volume of 0.1 ml were incubated at 35° C for 10 min unless otherwise stated. The reaction was stopped by immersion in an ice-water bath. The samples were rapidly transferred to filter paper strips and immersed in cold 15% trichloroacetic acid containing 1 μM ATP. The samples were heated for 10 min at 90—95° C, washed three times with 5% trichloroacetic acid, then with 95% ethanol, an ethanol/ether mixture (1 : 1, v/v) and ether. Radioactivity was measured by liquid scintillation spectrometry (Intertechnique ABAC SL/40).

Endogenous ^{32}P incorporation for each enzyme preparation was determined in the absence of substrate under the standard conditions. These values were substracted from total incorporation with substrate.

Assay of cyclic AMP binding activity

Binding of cyclic [^{3}H]AMP to protein was determined using a membrane filter assay in a total volume of 0.4 ml in the presence of 25 pmol of cyclic [^{3}H]AMP (240 cpm/pmol). The incubation medium contained also 50 mM Tris buffer, pH 7.4; 6 mM 2 mercaptoethanol; 10% glycerol; 0.8 mM 2′3′-AMP and 0.8 mM 5′-AMP. The incubation was carried out for 5 min at 30° C and 25 min at 4° C. Then 2 ml of ice cold buffer F (50 mM sodium acetate, pH 4.5; 2 mM sodium phosphate; glycerol 10%) was added and the samples filtered on 0.45 micromillipore filters which had been presoaked in buffer E. Filters were washed three times with 1 ml of buffer F, dried and dissolved in omnifluor and analysed for radioactivity.

Glycerol density gradient centrifugation

We used the method of Martin and Ames [19] to determine the sedimentation coefficients of the kinases. The enzymes were layered over 5 ml of a 15—30% glycerol gradient prepared in 10 mM Tris · HCl, (pH 7.6), 100 mM NaCl and 1 mM dithiothreitol. Centrifugation was for 24 h at 165 000 × *g*. Fractions each of 4 drops were collected and 60 μl aliquots were assayed for protein kinase activity as described previously using either histone or casein as substrate.

Results

Partial purification of protein kinases from plasmacytoma microsomes

The microsomal 0.6 M KCl wash was prepared from plasmacytoma microsomes and chromatographed on DEAE cellulose as described in Materials and Methods. Protein kinase activity was determined as the incorporation of ^{32}P from [γ-^{32}P]ATP into hot trichloroacetic acid insoluble material. Fig. 1 shows the elution profile of plasmacytoma microsomal protein kinase(s) from a DEAE cellulose column. The chromatography yielded one major, broad peak of protein kinase activity followed by a small shoulder which eluted between 0.05 and 0.18 M KCl when casein was used as substrate. This peak was pooled and chromatographed on a phosphocellulose column. Fig. 2 shows the elution profile from a P cellulose column of the major peak of protein kinase activity obtained after DEAE cellulose chromatography. In the absence of cyclic AMP and in the presence of casein two peaks of protein kinase activity were detected

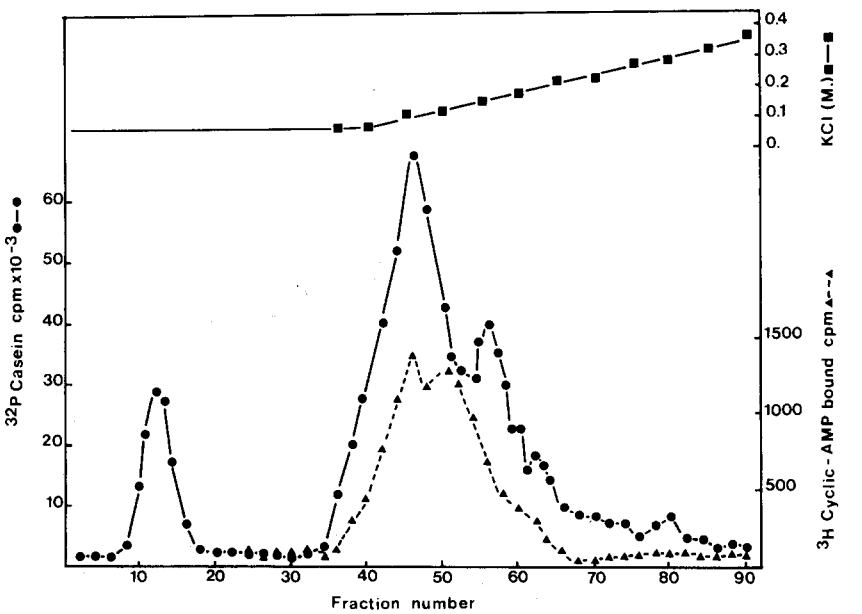

Fig. 1. DEAE cellulose chromatography of the microsomal KCl wash. About 65 mg of protein was chromatographed on a DEAE cellulose column as described in Material and Methods with a KCl gradient, (■———■). Fractions (3 ml) were collected and assayed for protein kinase activity using casein as substrate, (●———●) and for cyclic AMP binding, (▲- - - - -▲).

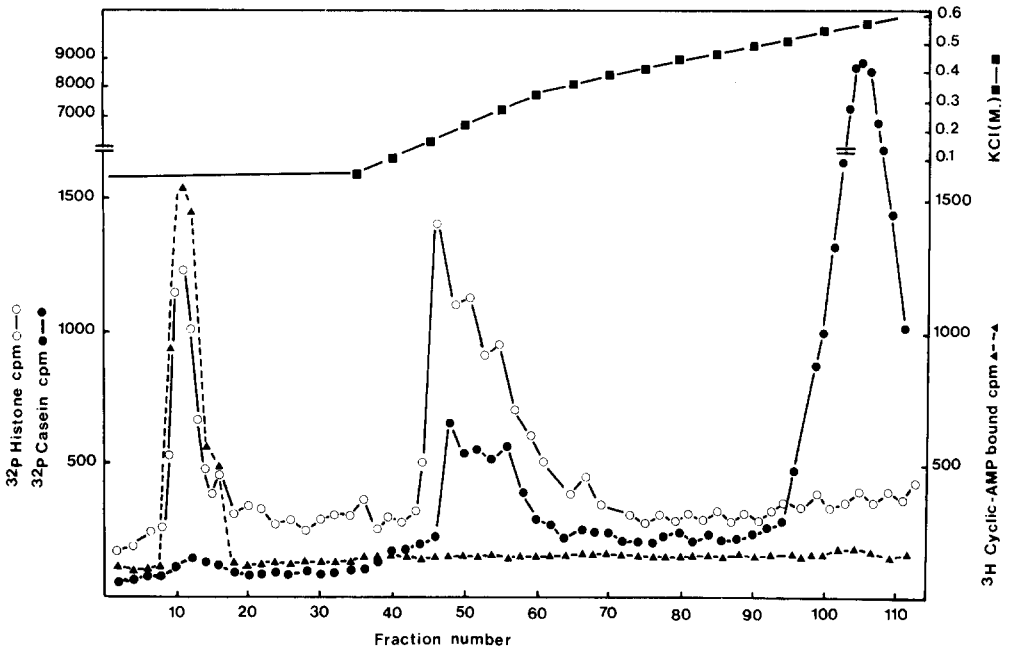

Fig. 2. P cellulose chromatography of the major peak of protein kinase activity eluted from the DEAE cellulose column (fractions 40—60). About 5 mg of protein was chromatographed on a P cellulose column as described in Material and Methods. Fractions (3 ml) were collected and assayed for protein kinase activity using either casein (●———●), or histone (○———○), as substrate and for cyclic AMP binding, (▲- - - - -▲).

(peak II and peak III) and in the presence of histone two peaks of activity were also observed (peak I and peak II). The different kinase peaks had been designated as protein kinase I, II and III in order of their elution from the P cellulose column.

Substrate specificity

Table I shows the various proteins tested as substrates for the different protein kinases obtained from plasmacytoma microsomes. In addition to histone, casein and ribosomal proteins as substrates, a partially purified protein fraction prepared from the ribosomal KCl wash was also assayed for its ability to serve as substrate. As we have shown in previous work [18] this fraction, obtained under conditions commonly employed for extraction of initiation factors, was phosphorylated both in vivo and in vitro by a ribosome-associated protein kinase and has been designated "PPx". It was eluted between 0.2 and 0.35 M KCl on a DEAE cellulose column [18] and here it was further purified by layering on a P cellulose column equilibrated in buffer E containing 0.1 M KCl. The study of the chromatographic behaviour on P cellulose of "PPx" was undertaken using an in vivo ^{32}P-labelled preparation of "PPx" prepared as described under Material and Methods. Fig. 3 shows that "PPx" did not adsorb P cellulose and passed through the column. An unlabelled preparation of "PPx" which eluted in the void volume of the P cellulose column was pooled, concentrated by Diaflo ultrafiltration (UM 10) and stored under liquid nitrogen. This

TABLE I

SUBSTRATE SPECIFICITY OF DIFFERENT RIBOSOMAL PROTEIN KINASES

Protein kinases (I, II, III) were prepared by DEAE cellulose and P-cellulose chromatography as illustrated in Figs. 1 and 2. Protein kinase activity was determined as described in Material and Methods using 40 μg of histone, casein. "PPx" protein(s) or 70 μg of ribosomes as substrate. The ribosomes were washed with 0.88 M KCl in buffer B, centrifuged and resuspended in the incubation medium. Values given were corrected for endogenous incorporation of enzyme.

^{32}P incorporated nmol/mg protein

Substrate	Protein kinases		
	I	II	III
Histone	2.87	6.32	0
Casein	0	0.85	27.5
"PPx" protein(s)	0	3.05	0
Ribosomal proteins	0	0	19.3

"PPx" fraction was used as substrate for protein kinase I, II and III. Table I shows the degree of phosphorylation of the different proteins. Total histone was found to be the only substrate of protein kinase I whereas casein and ribosomal proteins were excellent substrates of protein kinase III. Protein kinase II has a broader substrate specificity as it phosphorylates histone and the protein(s) of "PPx" at an appreciate rate while casein was a poor substrate. The 80 S ribosomal proteins were efficient substrates for only protein kinase III. The

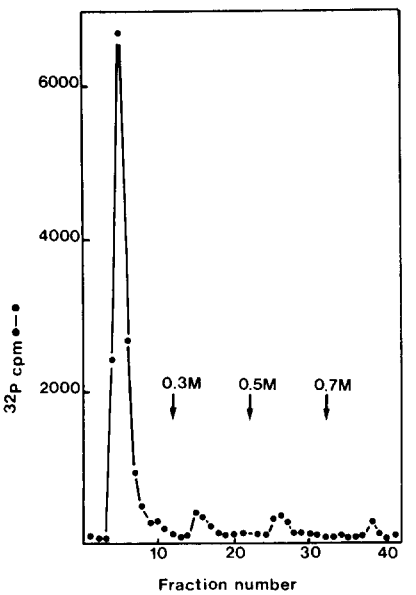

Fig. 3. P cellulose chromatography of a ^{32}P-labelled ribosomal "PPx" fraction obtained after DEAE cellulose chromatography. 1.4 mg of protein was applied on a 15 X 0.9 cm P cellulose column equilibrated in buffer E and eluted with a stepwise KCl gradient in buffer E. Fractions (2 ml) were collected and samples of 1 ml were used for the determination of radioactivity, (●———●).

TABLE II

COMPARISON OF KINETIC CONSTANTS OF VARIOUS PROTEIN SUBSTRATES FOR THE DIFFERENT RIBOSOMAL PROTEIN KINASES

The reactions were carried out under standard assay conditions. The protein substrate concentration varied between 20 and 120 μg/0.1 ml. V is expressed as nmol ^{32}P/mg enzyme; K_m as mg/ml.

Protein substrate	Protein kinases					
	I		II		III	
	V	K_m	V	K_m	V	K_m
Histone	23.4	2.42	9.4	0.15	—	
Casein	—		2.29	0.69	61.4	0.88
"PPx" protein(s)	—		20.0	2.3	—	

comparison of kinetic constants of the different protein substrates for protein kinase I, II and III is shown in Table II. "PPx" protein(s) which has high V and a relatively high K_m is a good substrate for protein kinase II. With casein the K_m value for both protein kinase II and III are similar, however the V for protein kinase II is much lower than the V for protein kinase III. With histone the K_m is lower for protein kinase II than for protein kinase I.

Nucleotide specificity

As several authors have reported that [γ-^{32}P]GTP can serve also as a phosphorylating donor for some protein kinases [3—5,20], we looked for the ratio of ATP/GTP utilization for protein kinase I, II and III. As shown in Table III, no kinase is specific to the phosphoryl donor, all three enzymes can utilize both ATP and GTP. However, under the standard conditions using equimolar concentration of GTP and ATP, ATP was a much better substrate than GTP for each type of protein kinase whatever substrate was used. Moreover, GTP serves as a better phosphoryl donor when histone and "PPx" protein(s) were used as

TABLE III

COMPARISON OF [γ-^{32}P]ATP AND [γ-^{32}P]GTP AS PHOSPHORYLATING AGENTS

Aliquots of the pooled fractions from P-cellulose column were assayed for protein kinase activity as described in Material and Methods using 80 μg of histone, casein or "PPx" protein(s), ATP and GTP were used at 0.15 mM.

^{32}P incorporated nmol/mg protein

Substrate	Nucleotide	Protein kinase fraction		
		I	II	III
Histone	+ATP	6.28	8.21	0
	+GTP	1.77	1.59	0
Casein	+ATP	0	1.13	49.50
	+GTP	0	0.24	0.61
"PPx" protein(s)	+ATP	0	5.16	0
	+GTP	0	1.97	0

substrate, while it is less efficient with casein and was therefore a very poor phosphoryl agent for protein kinase III.

Interaction with cyclic AMP

The major peak of protein kinase activity resolved by DEAE cellulose chromatography and the three peaks of kinase activity resolved by P cellulose chromatography were assayed for the binding of cyclic [^{3}H]AMP by the millipore filter technique as described under Material and Methods. It appears (Fig. 1) that binding activity for cyclic AMP was detected in the major peak from DEAE cellulose. After chromatography on P cellulose cyclic AMP bound only to protein kinase I whereas no cyclic AMP was retained by protein kinase II and III. This binding did not require Mg^{2+}. Moreover, when protein kinase I, II and III were assayed under the standard incubation conditions in the presence of increasing concentrations of cyclic AMP, there was no significant stimulation of incorporation of ^{32}P into protein substrates with protein kinase II and III, whereas a 2-fold stimulation was obtained with Protein kinase I and $5 \cdot 10^{-6}$ M cyclic AMP (Table IV).

Sedimentation of the protein kinases on glycerol density gradient

The sedimentation pattern of protein kinase I, II and III in a 15—30% glycerol gradient is shown in Fig. 4. Glycerol gradient centrifugation was carried out according to the procedure described by Martin and Ames [19]. In the absence of cyclic AMP in the gradient protein kinase activity of protein kinase I (assayed in the standard incubation medium in the presence of histone and $5 \cdot 10^{-6}$ M cyclic AMP) sedimented as one peak of about 4.4 S (Fig. 4A). When the sedimentation of protein kinase I was carried out after adding to the kinase $5 \cdot 10^{-6}$ M cyclic AMP, the glycerol gradient containing $5 \cdot 10^{-6}$ M cyclic AMP, the kinase activity was shifted to a lighter position of 2.6 S (Fig. 4B). This seems to indicate that in the presence of cyclic AMP, the heavy form of protein kinase I which possesses probably the regulatory (R) and catalytic (C) subunits had dissociated into a lighter component possessing enzymatic activity (C) in the absence of cyclic AMP.

The sedimentation of protein kinase II (Fig. 4C) exhibited two distinct peaks of protein kinase activity in presence of histone as substrate, a major peak of about 4.3 S and a small heavy peak of about 11 S.

TABLE IV

EFFECT OF CYCLIC AMP ON PROTEIN KINASE ACTIVITY

Aliquots of the pooled fractions from P cellulose column were assayed for protein kinase activity as described in Material and Methods using 80 μg of histone as substrate (protein kinase I and II) and 50 μg of casein (protein kinase III) in the absence or presence of $5 \cdot 10^{-6}$ M cyclic AMP.

Protein kinase fraction	^{32}P incorporated nmol/mg enzyme		
	—cyclic AMP	+cyclic AMP	Activity ratio: +cyclic AMP/—cyclic AMP
I	1.1	2.1	1.92
II	4.3	4.1	1
III	28	27.5	1

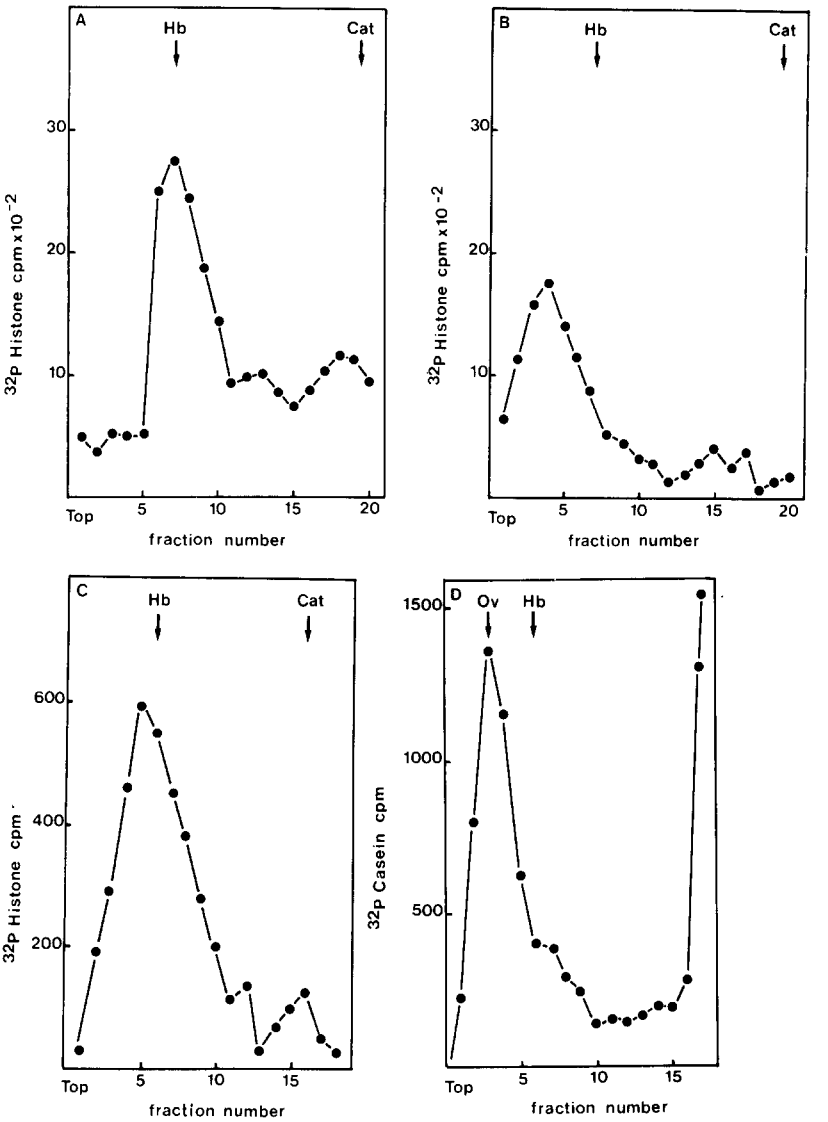

Fig. 4. Glycerol gradient centrifugation of protein kinases I, II and III. Protein kinase I (A and B), protein kinase II (C) and protein kinase III (D) after P cellulose chromatography were centrifuged on a 15—30% linear glycerol gradient under conditions as described in Material and Methods. Fractions (0.2 ml) were collected and 60 µl samples were assayed for protein kinase activity using histone (A, B, C) or casein (D) as substrate. (B): same as (A) except that the enzyme (protein kinase I) was treated with $5 \cdot 10^{-6}$ M cyclic AMP before centrifugation in 10^{-6} M cyclic AMP. Arrows indicate migration of ovalbumin (Ov), hemoglobin (Hb) and catalase (Cat) as standards.

The kinase activity of protein kinase III (Fig. 4D) sedimented as a 3.1 S single symetrical peak. Using hemoglobin (4.4 S, M_r 64 500); ovalbumin (3.5 S, M_r 46 000) and catalase (11.1 S, M_r 245 000) as standards the molecular weights of protein kinase I, II and III were roughly estimated to be respectively about 64 000 for protein kinase I, 60 000 for protein kinase II and 36 000 for protein kinase III.

Discussion

In this paper we have shown the occurrence of at least three different ribosomal protein kinases (protein kinase I, II and III) in plasma tumor cells. They have been isolated from the microsomal fraction of plasmacytoma and separated by chromatography on DEAE cellulose and P cellulose. Protein kinases have been classified into three groups by Traugh and Traut [5] where type I, type II and type III refer respectively to cyclic AMP dependent enzyme (holoenzyme "R · C" where R and C represent respectively the Regulatory and Catalytic subunit); to free catalytic subunit (C) and other protein kinases including the casein specific kinase. According to this classification protein kinase I from plasmacytoma ribosomes shows properties such as histone specificity, interaction with cyclic AMP, stimulation by cyclic AMP and behaviour on glycerol gradient centrifugation which are consistent with the characteristics of the protein kinase type I. This type of enzyme sedimented in a linear gradient without cyclic AMP as a 4.4 S component, whereas if the enzyme was in presence of $5 \cdot 10^{-6}$ M cyclic AMP and the gradient running in 10^{-5} M cyclic AMP all the activity then sedimented as a 2.6 S component indicating complete disssociation of the R · C complex. Moreover protein kinase I exhibits properties analogous to cyclic AMP dependent protein kinases isolated from the cytosol of other tissues [4—7,22].

The second peak of protein kinase activity (protein kinase II) has several interesting features: (1) it is rather tightly bound to ribosomes and cannot be removed by extraction with a low concentration of buffered KCl 0.1—0.4 M (data not shown). (2) It is the only enzyme which phosphorylates to a good extent the protein(s) of the ribosomal KCl wash fraction "PPx". (3) The enzyme is more active with histone and the "PPx" protein(s) than with casein. The histone/casein ratio is 7.5. The "PPx" protein(s)/casein ratio is 4.5. Both ATP and GTP serve as phosphoryl donors with a greater ratio of GTP/ATP utilization for "PPx" proteins. (5) The activity is not stimulated by cyclic AMP. (6) Protein kinase II is able to bind to plasmacytoma messenger RNA [23].

One could expect that protein kinase II, an enzyme which is fully active in the absence of cyclic AMP, would represent the free catalytic subunit of the R · C complex of protein kinase I. But if this is true protein kinase I in the presence of cyclic AMP would have phosphorylated "PPx" protein(s) and also casein; but this was not the case. Another possibility is that protein kinase II could be a catalytic subunit derived from the cytosol, but as we have shown previously [18] no protein kinase from the plasmacytoma cytosol was able to phosphorylate "PPx" protein(s). This data suggest that the ribosomal and soluble protein kinases are not related. Moreover, the results obtained cannot rule out the possibility that in vivo protein kinase II would be cyclic AMP dependent and present in a more labile R · C form than protein kinase I. So under our extraction conditions using high ionic strength (0.6 M KCl) this form of enzyme would dissociate, liberating the free catalytic subunit protein kinase II.

Protein kinase III enzyme which was not stimulated by cyclic AMP and was highly casein specific could be of type III. It resembles the casein kinase III

isolated from the cytosol of reticulocyte [5] but it differs markedly from it because for the ribosomal protein kinase III, GTP had a very low efficiency as phosphoryl donor while for soluble casein kinase IIIc GTP was two-thirds as effective as ATP. As our method of microsome isolation involved sedimentation of microsomes in buffered sucrose at low ionic strength (0.025 M KCl) we thought that most if not all of the protein kinase I and III kinase activities were contaminants from the cytosol as it has been shown to occur in rats' hearts by Kealy et al. [21]. Therefore, we recently analysed the elution profil from a P cellulose column of protein kinases obtained from purified free polysomes (without any contamination of endoplasmic reticulum) and we found that it was similar to that given by the microsomal protein kinases (to be published). So it appears that all three types of protein kinases isolated from microsomes were associated to ribosomes.

Nevertheless the highly "PPx" specific kinase, protein kinase II, seems to be the most interesting ribosome-bound protein kinase as it has not been found in the cytosol of plasmacytoma [18]. The main characteristics of protein kinase II are in agreement with the observations of Jergil [8] and Fontana et al. [9] who described also a ribosome-bound kinase in trout testis and reticulocyte ribosomes. However, in view of our results their preparation of ribosomal protein kinase seems to be more or less contaminated by enzyme types I and III.

We have shown previously that at least two proteins of the "PPx" fraction (M_r about 90 000 and 115 000) were phosphorylated by the ribosome associated protein kinase [18] which corresponds to protein kinase II. As these proteins have been isolated from the ribosomes by high ionic strength, conditions commonly employed for extraction of initiation factors, it is tempting to postulate that phosphorylation of some initiation or preinitiation factors may play a role in the regulation of eukaryotic protein synthesis.

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