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THE PROTEIN KINASES OF RAT LIVER NUCLEI

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SUMMARY

Soluble proteins of rat liver nuclei were applied to a DEAE-Sephacel column and eluted with a linear gradient of 70-600 mM NaCl. Three major classes of nuclear protein kinase were identified. There were types I and II cAMP-dependent protein kinase, a Ca-calmodulin-dependent protein kinase, and a third enzyme which was stimulated by cAMP-dependent protein kinase inhibitor.

The patterns of PK (protein kinase) activity and nuclear protein phosphorylation change as cells pass through their growth-division cycle (1-3). There are also several lines of evidence suggesting that PKs may play a key role in the initiation of DNA synthesis. Thus, for example, hepatocytes in regenerating liver and cultivated non-neoplastic cells need both calcium and a brief cAMP surge in late G1 phase to initiate DNA synthesis (4). Moreover, cultivated liver cells arrested in late G1 phase by calcium deprivation can be induced to initiate DNA synthesis, almost immediately by calcium, cAMP, or type II cAMP-dependent PK (5).

As few as two and as many as twelve forms of PK with differen cation requirements and substrate preferences have been found in nuclei (6-10), but the presence of cAMP-dependent PKs in the nucleus has always been controversial (3,7,9,11). However, cAMP-dependent PK activity has recently been found in thymic lymphocyte

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nuclei (12). In this communication we report the presence of several types of PK in rat liver nuclei, including two types of cAMP-dependent PK, and a hitherto unnoticed PK which is stimulable by Ca-calmodulin.

MATERIALS

Bovine serum albumin, cAMP, cGMP, calf thymus histones Sigma type IIA (mixed) and Sigma type V-S (H1), beef heart protein kinase inhibitor (PKInh), salmon sperm protamine, 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); DEAE-Sephacel was obtained from Pharmacia (Uppsala, Sweden); [2,8-^{3}H]cAMP (37 mCi/mmole) was obtained from Amersham Corp. (Oakville, Ontario, Canada). [γ-^{32}P]ATP was prepared according to Glynn and Chappell (13), and calmodulin was purified to homogeneity from rat testis according to MacManus (14).

METHODS

Isolation of nuclei and preparation of soluble nuclear protein extract.

Liver tissue from specific pathogen-free male Sprague-Dawley rats (190-210 g) was homogenized in 6 volumes of buffer A (50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, pH 7.5) and then centrifuged at 700 g_{av} for 10 min at 2°C. The pellet was washed with buffer A, and resuspended in 5 volumes of buffer A containing 1% Triton X-100. After centrifugation the pellet was washed again with buffer A.

The nuclear preparations were slightly contaminated by mitochondria (about 5% of total mitochondrial monoamine oxidase and succinic dehydrogenase activity was associated with nuclei), but contained neither lysosomes (assessed by fluorescence microscopy after staining with acridine orange) nor microsomes (indicated by no NADPH cytochrome c reductase activity).

Nuclei were extracted with 0.6 M NaCl in buffer B (20 mM Tris-HCl, 3 mM $MgCl_2$ 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 7.5). The nuclear suspension was centrifuged for 15 min at 20,000 g_{av}, the pellet was discarded, and the supernatant diluted 10-fold with buffer B. The precipitate formed in the diluted supernatant was removed by centrifugation at 10,000 g_{av} for 10 min, and the clear supernatant containing 50% of the original nuclear proteins and about 80% of the total nuclear PK activity was applied to a DEAE-Sephacel column, as described in the figure legends.

PK (Protein kinase) assay

PK activity was assayed according to Takai et al. (15). The assay mixture (0.25 ml) contained: 2.5 nmole [γ-^{32}P]ATP (2×10^5 cpm/nmole), 7.5 µmole 2-mercaptoethanol, 1.25 or 18.75 µmole $MgCl_2$, 5 µmole Tris-HCl (pH 7.5), 100 µg of mixed histone, histone H1 or protamine, and the appropriate enzyme fraction. The mixture was incubated for 5 min at 30°C, and the reaction was stopped by adding 20% trichloroacetic acid (TCA). Bovine serum albumin (0.5 mg) was added as a carrier. The acid-precipitable material was washed three times by dissolving in 1.0 N NaOH and re-precipitating in TCA. The radioactivity of ^{32}P incorporated

into the protein substrates was measured with a Beckman LS 255 liquid scintillation counter.

Other procedures

cAMP-Sepharose was prepared as described by Dills et al. (16). The coupled density of immobilized N^b-$H_2N(CH_2)_2$-cAMP was 6 µmole/ml of settled gel. The regulatory subunit of cAMP-dependent PK attached to this gel was isolated and purified by elution with 1 mM cGMP, concentration on an Amicon filter (XM5) and extensive dialysis.

Protein content was measured by a dye-binding assay (Bio-Rad: 17).

RESULTS AND DISCUSSION

The cytoplasmic extracts of liver cells contained the two classical forms (I and II) of cAMP-dependent PK, which were eluted from DEAE-Sephacel by 100 and 200 mM NaCl respectively (18). They

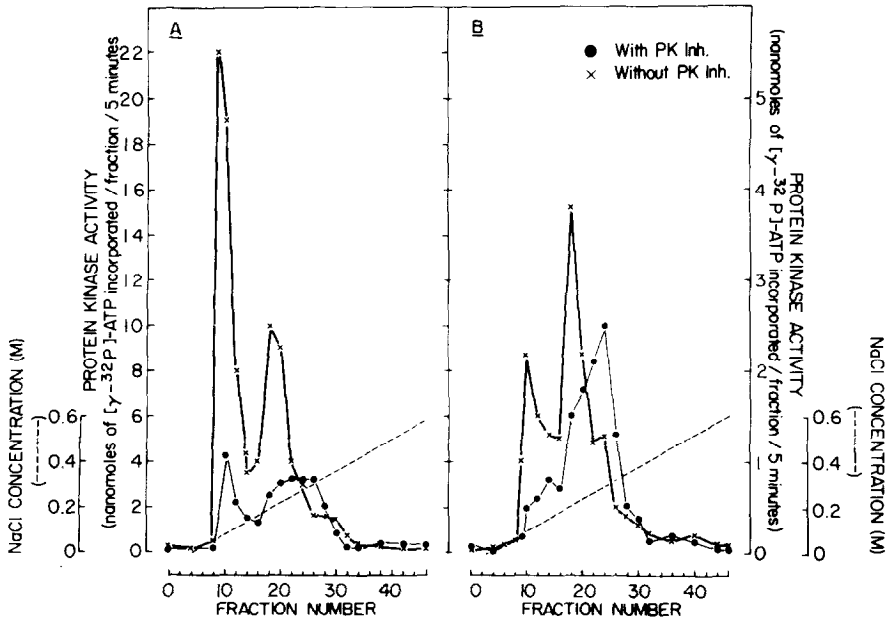

Figure 1. The protein kinases from the cytoplasm and nuclei of liver cells.

40 mg of cytoplasmic (A) or nuclear (B) proteins were applied to DEAE-Sephacel columns (0.9 × 30 cm) previously equilibrated with buffer B. The column was washed with the same buffer, and elution was carried out with 100 ml of a linear gradient of NaCl (0. - 600 mM), and 2 ml fractions were collected.

Enzymatic activity was measured with 5 mM Mg^{2+}, mixed histones, 10^{-6}M cAMP, 2 mM IBMX, and with or without 10 µg of protein kinase inhibitor (PKInh).

phosphorylated mixed histones in the presence of 5 mM Mg^{2+}, and were inhibited by a specific cAMP-dependent PK inhibitor, PKInh (Fig. 1A). On the other hand, there were three distinct peaks of PK activity in nuclear extracts, two of which were eluted by the same NaCl concentrations as the cytoplasmic enzymes and, like the latter, were inhibited by PKInh (Fig. 1B). The third type of nuclear activity was eluted by 300 mM NaCl and was stimulated by PKInh (Fig. 1B).

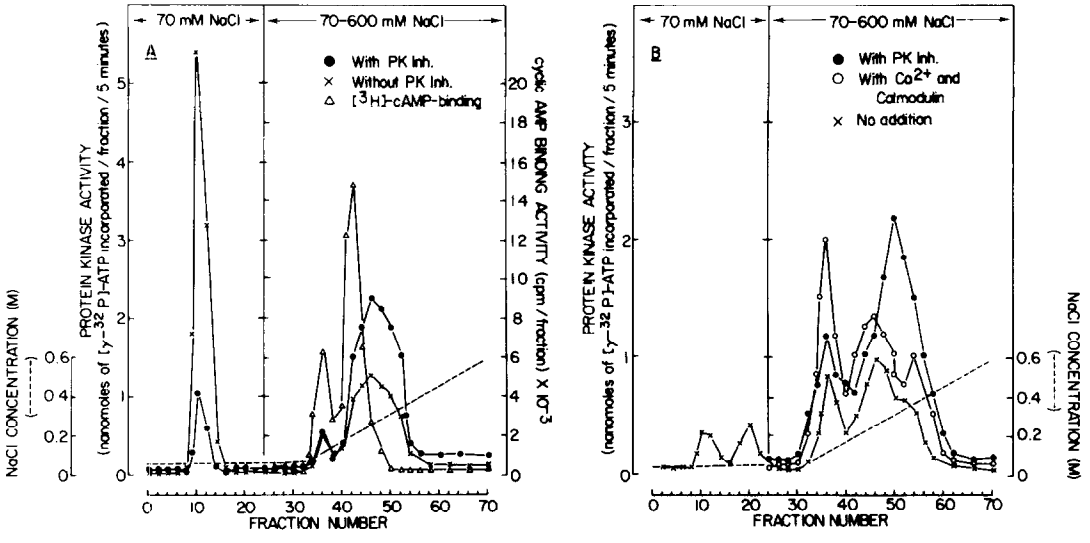

Figure 2. DEAE-Sephacel chromatography of soluble liver nuclear protein kinases treated with cAMP.

Nuclear proteins (12 mg) were applied to a DEAE-Sephacel column (0.9 × 30 cm) equilibrated with buffer B. After the column was washed, the first elution was carried out with 50 ml of 70 mM NaCl and 10^{-5}M cAMP, followed by the second elution with 100 ml of a linear concentration gradient of NaCl (70 - 600 mM), and 2 ml fractions were collected.

To establish cAMP-binding activity, nuclear proteins were loaded on to the column in the presence of 1 nmole of [^{3}H] cAMP (37 mCi/mmole). Before the first elution, the column was washed extensively with buffer B. PK activity was assayed with 5 mM Mg^{2+}, mixed histone substrate, 2 mM IBMX, 10^{-6}M cAMP, with or without 10 µg of PKInh (panel A), or (panel B) with 75 mM Mg^{2+}, protamine substrate, with or without 10 µg of PKInh. In panel B, the fractions were also assayed in the presence of 3 × 10^{-6}M calmodulin and 5 × 10^{-5}M Ca^{2+} (panel B).

If the two PKInh-inhibitable activities were due to two cAMP-dependent PKs, they should have been stimulable by cAMP, which would dissociate them into two distinct regulatory (R) subunits and two nearly identical active catalytic (C) subunits (18). However, their activities were not stimulated by addition of cAMP to the appropriate fractions, probably because they were altered by the high NaCl concentration used to extract them (12). On the other hand, cAMP treatment of the nuclear extract (as described in the legend of Fig. 2) generated a new peak of PKInh-inhibitable activity, which was eluted by 70 mM NaCl (Fig. 2A) and was probably due to liberated C subunits which preferred to phosphorylate histones in 5.0 mM Mg^{2+} (Table 1). As expected, two types of cAMP-binding protein remained on the columns which

TABLE 1
Magnesium requirements and substrate preferences of the three major nuclear classes of PK

Substrate	Protein Kinase activity (%)			
	Magnesium concentration (mM)			
	5	20	50	75
	Fraction 10 (C subunit of cAMP-dependent PK)			
Histone, mixed (type IIA, Sigma)	100	75	31	21
Histone, H1 (type V-S, Sigma)	60	51	20	13
Protamine	11	10	8	7
	Fraction 36 (Ca-calmodulin stimulable PK)			
Histone, mixed (type IIA, Sigma)	100	92	73	70
Histone, H1 (type V-S, Sigma)	61	41	26	25
Protamine	108	80	114	175
	Fraction 50 (PKInh-stimulable PK)			
Histone, mixed (type IIA, Sigma)	100	81	56	55
Histone, H1 (type V-S, Sigma)	108	107	38	27
Protamine	50	64	60	65

PK activity is expressed as the percentage of that assayed with mixed histones (in 5 mM magnesium) as a substrate.

TABLE 2
Effects of cAMP and regulatory (R) subunit on the phosphorylating activity of catalytic (C) subunit.

	phosphorylating activity cpm ^{32}P from [γ-^{32}P]ATP/100 µg mixed histones/5 min
C subunit*	16,860
R subunit**	1,200
(C + R) subunits	9,960
(C + R) subunits + cAMP	15,150

* Extensively dialyzed fraction 10 (Fig. 2A) was the source of C subunit

** Fractions 36-46 (Fig. 2A) were pooled and R subunits were purified on a cAMP-Sepharose affinity column

were eluted with 120 and 220 mM NaCl (Fig. 2A) and, when further purified (on cAMP-Sepharose column), substantially reduced the activity of the putative C subunits from fraction 10 (Fig. 2A). This reduction was overcome, in turn, by re-adding cAMP (Table 2) Thus, it seems that the cytoplasm and nuclei of non-proliferating rat liver cells contain different proportions of the same two forms of cAMP-dependent PK.

The high activities of the cAMP-dependent PKs masked those of the minor PKs eluted by the same range of NaCl concentrations. However, these minor activities were revealed when the putative C subunits of the cAMP-dependent PKs were eluted with 70 mM NaCl and 10^{-5} M cAMP. Two groups of enzyme were detected in the fractions eluted from the gradient by higher NaCl concentrations. The first group was eluted by 100-150 mM NaCl, peaked in fraction 36, and was seemingly unaffected by PKInh (Fig. 2A), while the second one, which peaked in fraction 46, was strongly stimulated by PKInh and was probably the one responsible for the third peak of activity in Figure 1B. The activity profile was similar when assayed in 75 mM Mg^{2+} with protamine as a substrate, but the activities were higher in fractions 32-40 and lower in fractions 46-52 (Table 1, Fig. 2B). Although fractions 46-58 were still

TABLE 3
Activation of nuclear PK(s) from fraction 36 (Fig. 2A) by calcium and calmodulin

	Protein kinase activity pmoles ^{32}P from [γ-^{32}P]ATP/2 ml of fraction 36/5 min		
	Protamine		Histones (mixed)
	10 mM Mg^{2+}	75 mM Mg^{2+}	10 mM Mg^{2+}
Basal activity	377	539	397
+ Ca^{2+} (5×10^{-5}M)	361	510	317
+ Calmodulin (10^{-6}M)	476	596	450
+ Ca^{2+} + calmodulin	834	1787	798
+ Ca^{2+} + calmodulin + EGTA (5×10^{-4}M)	327	-	377

significantly stimulated by PKInh with protamine as substrate in 75 mM Mg^{2+}, the larger peak of PKInh-stimulable activity was in fraction 50 (Fig. 2B) rather than fraction 46 (Fig. 2A).

Since a commercial PKInh preparation stimulated a cGMP-dependent PK (19-20), and since such an enzyme has been found in nuclei (21), the enzyme(s) stimulated by PKInh in this study might also have been cGMP-dependent. However, the maximally PKInh-stimulable enzyme(s) in fraction 50 was unaffected by 10^{-6}M cGMP when assayed with mixed histones in the presence of 5, 20, or 75 mM magnesium (data not shown).

There was a hitherto unnoticed PK species in fractions 32-40 (eluted with 80-120 mM NaCl), which was more active in 75 mM Mg^{2+} with protamine as substrate (Tables 1 and 3), and was strongly stimulated by Ca-calmodulin. Thus, 5×10^{-5} M calcium and 10^{-6} M calmodulin together (but not separately) increased the enzyme's activity 2- to 3-fold while the specific calcium chelator EGTA abolished this stimulation (Table 3).

We conclude that nuclei from proliferatively inactive liver cells contain three main classes of PKs, the activities of which are stimulated by cAMP, Ca-calmodulin or PKInh, and which have strikingly different magnesium requirements and substrate

preferences (Table 1). In view of the importance of calcium and cAMP in proliferative development, the synthesis and activation of at least some of these enzymes will almost certainly be found to play important roles at critical points in the growth-division cycle of liver cells.

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