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A 25 000 DALTON INHIBITOR OF cAMP INDEPENDENT PROTEIN KINASES PRESENT IN RAT LIVER HMG PROTEIN PREPARATIONS

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SUMMARY. A protein kinase inhibitor was found in rat liver cells as a component of HMG proteins. It is located in cytosol as well as in nuclei. It inhibits all tested cAMP independent protein kinases and has no effect on cAMP dependent protein kinases. This inhibitor is a 25 000 Da protein. It has no ATPase, phosphoprotein phosphatase or proteinase activity and is heat unstable.

Protein phosphorylations have been implicated in many regulatory mechanisms in nuclei as well as in cytoplasm and in membranes (for review, see 1). cAMP independent protein kinases are able to phosphorylate many different proteins, including histones and chromosomal non histone proteins. It has been shown that nuclear proteins are extensively phosphorylated in metabolically active cells by endogenous protein kinases (2-4). However, very little is known about the regulation of the activity of these enzymes. Several protein kinase inhibitors of various chemical nature, which may play a regulatory role in phosphorylation, have been described in the nuclei and the cytosol : some are proteins (5 7), glycoaminoglycans (8,9) heparin (10,11), oligonucleotides (12), quercitin and related polyphenols (13), and polyglutamyl peptides (14). Others are of unknown nature (15-18). Some of them act on cAMP independent protein kinases : nuclear protein kinases (12,15), cytosolic casein kinase type II (8,14,16,17), cytosolic casein kinases type I and II (18) and others on cAMP dependent protein kinases (6,17).

In the course of our study of HMG protein phosphorylation, we observed that an extract containing HMG proteins strongly inhibits the phosphorylation of other chromosomal non histone proteins. This inhibitor

Abbreviation : **HMG proteins = high mobility group proteins.**

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copurifies with HMG proteins and is located in cytosol as well as in nuclei. It is specific for cAMP independent protein kinases.

MATERIALS AND METHODS

Preparation of the inhibitor. The inhibitor is present in the HMG crude preparations. **(a) From whole liver.** Livers from Wistar male rats weighing 180 200 g, fasted for 36 h were minced and homogenized using a Thomas Potter, in 0.5 ml of 0.75 M perchloric acid per g of liver. The very dense homogenate was centrifuged for 20 min in a RC2B Sorvall centrifuge at 15 000 g. **(b) From rat liver nuclei.** Nuclei were isolated from the rat livers according to Chauveau **et al** (19) and the washed nuclei were treated with perchloric acid as described above. **(c) From rat liver cytosol.** Rat livers were homogenized using a Thomas Potter in 0.25 M sucrose, 10 mM Tris (pH 7.5), 3.3 mM $CaCl_2$. The homogenate was centrifuged for 20 min at 15 000 g in a RC2B Sorvall centrifuge. The supernatant was centrifuged in a Beckman 50 Ti rotor at 150 000 g for 60 min. Perchloric acid was added to the supernatant to a final concentration of 5%. Insoluble material was discarded after 20 min of centrifugation in a RC2B Sorvall centifuge at 15 000 g.

Protein kinase preparation. (a) Nuclear cAMP independent protein kinases NI and NII. The protein kinases were partially purified from nuclear extracts including non histone proteins by affinity chromatography on a casein-phosvitin-Sepharose column (unpublished technique). This procedure completely removes the inhibitor, and fractions were obtained containing protein kinases and part of their substrates. **(b) DNA-bound cAMP independent protein kinase.** We have recently described a protein kinase tightly bound to DNA (20). It was prepared from rat liver nuclei. The protein kinase was released from the 0.45 M NaCl insoluble material by digestion with 2.5 mg DNase I, 5 mM $MgCl_2$ for 6 h at 20°C. **(c) Cytosol cAMP independent protein kinases CKI and CKII.** The enzymes were partially purified from cytosol using the technique described for the nuclear protein kinases NI and NII. **(d) Cytosolic cAMP dependent protein kinase.** Acetic acid was added to cytosol to give a pH of 4.8. The precipitate was discarded by centrifugation. The supernatant was neutralized to pH 7.0 with 1 M Tris-HCl. The protein kinases were precipitated by ammonium sulfate to a final 65% concentration. The precipitate was dissolved in water and extensively dialyzed against 0.1 M Tris-HCl (pH 7.8).

Protein kinase assays. The incubation medium included in a 250 µl final volume : 100 µg of partially purified protein kinase preparation which includes some of the endogenous substrates and the enzymes, 5 µCi (γ-^{32}P) ATP (3 Ci/mmole Amersham), 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$. Incubation was carried out for 60 min at 37°C. The proteins were precipitated with 1 ml of 33% trichloroacetic acid. The precipitates were collected on Millipore HAWP 0.45M filters. The filters were washed with 10 ml of 25% trichloroacetic acid, transferred to 5 ml of Unisolve and the radioactivity measured. One unit of protein kinase is the amount of enzyme which catalyzes the transfer of 1 pmole of ^{32}P from ATP to substrate per min under standard assay conditions.

Inhibitor assay. Inhibitor activity was measured by adding the inhibitor to a standard protein kinase assay. One unit of inhibitor is the amount that reduces the activity of 1 unit of protein kinase to 50% of its initial value.

Phosphatase protease and ATPase activity assays. The incubation medium was prepared as described above. After 10 min of incubation at 37°C in the presence of 3 Ci/mmol of (γ-^{32}P ATP), the medium was separated into 3 parts of identical volumes : the inhibitor and a large excess of unlabelled ATP (final concentration 100 µM) were added to the first part, non radioactive ATP alone was added to the second part, 10 mM Tris was added to the third part. Aliquots were taken after various times of incubation at 37°C and the

incorporated radioactivity was measured. The ATPase activity was measured using the activated Norit charcoal method of Lowery and Richardson (21).

Molecular weight determination. Molecular weight was estimated by high performance liquid gel filtration (22). A LKB TSK G 3000 W 7.5x600 mm column was used with the following buffer : 0.1 M NaCl, 10 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$ at a flow rate of 1 ml/min. Fractions of 0.6 ml were collected. The column was calibrated with the Boeringer molecular weight markers kit comitek TM. The presence of inhibitor was tested in each fraction using partially purified NI-NII protein kinases.

Electrophoreses. Electrophoreses were performed according to Laemmli (23) with sodium dodecyl sulfate-gradient polyacrylamide gel, from 7.5 to 20%.

RESULTS

Evidence for a protein kinase inhibitor in rat liver perchloric acid extract. A crude HMG protein preparation was obtained from rat liver by perchloric acid extraction followed by acetone precipitation. Chromosomal non histone proteins were prepared from rat liver, and included cAMP independent protein kinases type NI and NII and non histone protein substrates of these protein kinases. The addition of the perchloric extract to the chromosomal non histone proteins strongly inhibited the endogenous protein kinases (Fig.1). It may be concluded that a cAMP independent protein kinase inhibitor is present in the crude HMG protein preparation.

Characterization of the inhibitor. Thermal stability. Preincubation of the inhibitor preparation for 10 min at 60°C completely suppressed its inhibitory activity. **Effect of proteolysis.** Incubation of the preparation with 1 mg/ml of proteinase K for 15 min at 37°C followed by re-extraction with 0.75 M perchloric acid and dialysis completely suppressed the inhibitory activity. The same treatment performed in the absence of proteinase K reduced the inhibitory activity approximately 50%. This reduction in inhibitory activity is likely to result from heat denaturation and loss of material. These observations strongly suggest that the inhibitor is a protein.

Essays of phosphoprotein phosphatase, protease and ATPase activities in the inhibitor preparations. A chase experiment was performed. After incubation of chromosomal non histone proteins with (γ-^{32}P) ATP for 10 min at 37°C, the incubation medium was supplemented with the inhibitor and an excess of non-labelled ATP. Fig.2 shows that this addition does not reduce the incorporated radioactivity. This experiment demonstrates that the inhibitor preparation does not contain phosphoprotein phosphatase or protease activities. The presence of ATPase activity in the preparation was tested using the activated charcoal technique. The charcoal bound and unbound radioactivities are not modified by incubation at 37°C in the presence of the inhibitor fraction for times ranging from 1 to 30 min, excluding the presence of an ATPase activity in the inhibitor fraction.

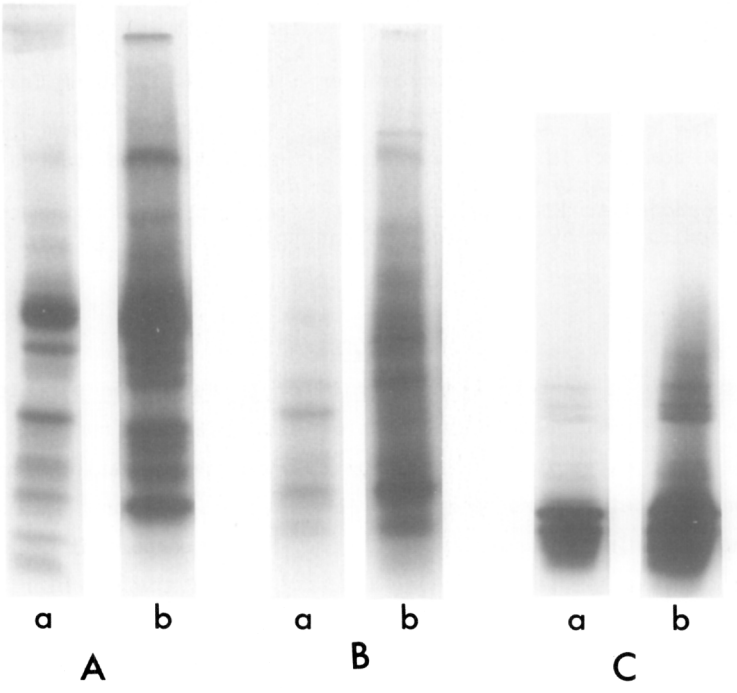

Figure 1. Effect of the inhibitor on the phosphorylation of natural substrates by cAMP independent protein kinases.

The protein kinases were prepared under such conditions that they remained associated with some of their natural substrates. They were incubated with (γ-^{32}P) ATP. A- Cytosolic protein kinases were incubated in the presence of 50 µg of inhibitor preparation (a) and in the absence of inhibitor (b). B- Nuclear protein kinases were incubated in the presence of 50 µg of inhibitor preparation (a) and in the absence of inhibitors (b). C- DNA-bound protein kinase was incubated as described above. After the incubation, the proteins were separated by electrophoresis performed according to Laemmli (23), and submitted to radioautography.

Apparent molecular weight. The molecular weight of the inhibitor was determined by gel filtration using a HPLC system. Inhibitory activity was determined in each eluted fraction using a mixture of purified nuclear NI and NII protein kinases. The inhibitory activity eluted as a single peak corresponding to an apparent molecular weight of 25 000 (not shown).

Determination of the protein kinases inhhibited by the inhibitor. The inhibitor was tested on cAMP independent protein kinases : nuclear protein kinases NI and NII, DNA-bound protein kinases and cytosolic protein kinases CKI and CKII. The substrates were the proteins present in the protein kinase preparations, casein and phosvitin. In all cases a strong inhibition was observed (Fig.1, Table I). No inhibition was observed when cytosolic cAMP dependent protein kinases were used.

Localization in the cell. Since HMG proteins have been found in nuclei and in cytosol (24), we have prepared the inhibitor from these two

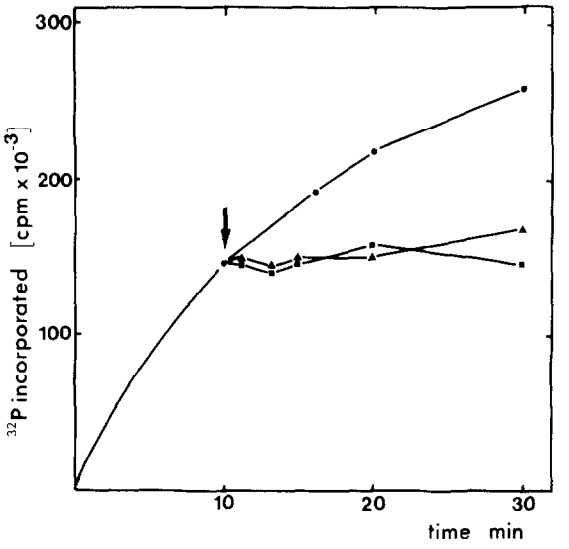

Figure 2. Effect of the inhibitor preparation on the incorporated (^{32}P) phosphate.

Three protein kinase assay media (1.5 ml) were incubated for 10 min at 37°C. One mixture was then supplemented with 250 µl of 0.1 M Tris-HCl (pH 7.1), 10 mM $MgCl_2$ (●—●), the second was supplemented with 0.2 mM ATP dissolved in the same solution (■—■), and the third was supplemented with the same medium including the excess of ATP and 200 µg of the inhibitor preparation (▲—▲). Aliquots were taken after various lengths of time and the incorporated radioactivities measured.

fractions. The inhibitor was found to be present in both cell fractions with no significant differences in activity.

DISCUSSION

An inhibitor specific for cAMP independent protein kinases which co-purifies with HMG proteins has been characterized. Its sensitivity to heat denaturation and to proteinase K strongly suggests that the inhibitor is a protein. Direct assays and chase experiments allowed us to eliminate the possibility that the inhibitor could act through an ATPase, a phosphoprotein phosphatase or a proteinase activity.

Its molecular weight, as estimated by HPLC gel filtration, is approximately 25 000, which is intermediate between those of HMG 2 and HMG 14 proteins. None of the major HMG proteins has an inhibitory effect on protein kinases. The possibility that the inhibitor is a minor HMG protein has not been excluded. The proteins of this class are characterized by a bipolar structure, with basic amino acid residues at the N terminal part of the molecule and acidic amino acid residues at the C terminal part (25). Recently Meggio **et al** (11) have shown that polyglutamic acids are efficient non-competitive inhibitors of cAMP independent protein kinase CK_{II} with a

Table I

Effect of the inhibitor on various protein kinases

Protein kinase	Added substrate or effector	Kinase activity in the absence of inhibitor u/mg	Kinase activity in the presence of 100 µg of inhibitor u/mg	Residual activity %
Nuclear protein kinases NI and NII crude preparation	0	2840	548	19
	phosvitin 100 µg	12170	2575	21
	casein 100 µg	7385	1350	18
DNA-bound protein kinase	0	63	24	38
Cytosolic protein kinases CKI and CKII crude preparation	0	3617	1482	41
	phosvitin 100 µg	12660	4350	34
	casein 100 µg	11580	4185	36
Cytosolic cAMP dependent protein kinases acid preparation (a)	0	97	117	120
	cAMP 10^{-5}M	112	146	130
	cAMP 10^{-5}M + Histone 100 µg	183	281	150
	cAMP 10^{-6}M	118	162	137
	cAMP 10^{-6}M + Histone 100 µg	185	300	161

(a) The increase in activity observed with the inhibitor is due to the presence in the inhibitor preparation of a small amount of histone H1 which is a substrate of these protein kinases.

K_i of 0.11 µM, which suggests that the inhibitor described in this paper could act through a similar mechanism.

The inhibitor acts in the presence of various substrates : chromosomal non histone phosphoproteins, cytoplasmic phosphoproteins, phosvitin and casein. It has no effect on cAMP dependent protein kinases (Table I).

The inhibitor described in this paper is different from the inhibitors previously described. The inhibitor described by Job **et al** (16) has a lower molecular weight, 16 500 and has a strict cytosolic localization, as it is the case for the enzyme described by Appelman **et al** (26). The inhibitor we have studied is different from the inhibitor described by Farron-Furstenthal (7,12,27), although both are active on cAMP independent protein kinases. However three major differences exist. The inhibitor

described by Farron-Furstenthal has (i) a molecular weight of 150 000 instead of 25 000 (ii) a strict specificity towards nuclear protein kinases and (iii) a strict nuclear localization. The inhibitor described in this paper is also different from that reported by Dabauvalle **et al** (15) which is also present among chromosomal non histone proteins but which is thermostable, is specific for N II protein kinase and has a very small molecular weight.

The inhibitor described in this paper, as well as the previously described inhibitors may regulate the nuclear phosphorylations, since the nuclear protein kinases are apparently not regulated by any other mechanism and also part of the cytoplasmic phosphorylations.

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