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INCREASED ACTIVITY OF RAT LIVER NUCLEOLAR PROTEIN KINASE FOLLOWING TRIIODOTHYRONINE ADMINISTRATION

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Summary

Triiodothyronine (T_3) administration to thyroidectomized rats induces a significant increase in the nucleolus-associated protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity. The general properties of the protein kinase solubilized from liver nucleoli have been investigated. Mg^{2+} (20 mM) is essential for the reaction and an appropriate concentration of NaCl (100 mM) is required to achieve maximal phosphorylation rates. The optimal pH for casein phosphorylation is 7.6. The kinase phosphorylates casein more efficiently than phosvitin and displays an almost undetectable activity towards histones and protamine. No significant stimulation of the kinase activity by cyclic AMP has been detected. The apparent K_m values for casein and ATP are 1.5 mg/ml and $1.5 \cdot 10^{-5}$ M, respectively, and are not affected by the hormone administration.

Introduction

In a previous work we reported that triiodothyronine (T_3) administration stimulates the in vitro phosphorylation of rat liver nucleolar proteins [1]. Such a stimulation is concomitant with the well known increase in the synthesis of pre-rRNA as well as in the activity of nucleolar RNA polymerase I which occurs in rat liver nucleoli as early as 12 h after T_3 injection [2,3].

Considerable evidence is now available supporting the view that the phosphorylation of chromatin-associated proteins plays a positive role in the control of transcription [4—6]. While the nuclear kinases which catalyze the phosphorylation of these proteins have been studied extensively [7—9], little is known about nucleolar protein kinase(s) [10—12].

The present work was undertaken (a) to delineate the general properties of

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protein kinase(s) associated to rat liver nucleoli and (b) to investigate whether the T_3-induced increase in the in vitro phosphorylation of rat liver nucleolar proteins depends on an enhanced protein kinase activity.

The data presented in this paper demonstrate that T_3 administration causes a significant increase in the nucleolus-associated protein kinase activity within a few hours. This increase is not related to enhanced catalytic efficiency but to a greater level of the enzyme.

Materials and Methods

Animals. Thyroidectomized male albino rats (Wistar strain) weighing 140—180 g were used. Surgical thyroidectomy was performed four weeks before the experiments, by which time their body weight was about 25—30% below the values of unoperated controls. The animals were fed on Purina Laboratory pellet ad libitum and were starved overnight prior to killing. 3,5,3'-Triiodo-L-thyronine (Fluka A.G., Buchs, Switzerland) was injected intraperitoneally (30 μg/100 g body weight) in isotonic alkaline saline at fixed times before death. Control rats received the same volume (0.5 ml/100 g body weight) of isotonic alkaline saline alone.

Preparation of nucleoli. Nucleoli were isolated from liver nuclei as previously described [3]. The purity of the nucleoli was assessed by the amount of nucleoplasmic DNA-dependent RNA polymerase II present as determined by assay of nucleoli in the presence and absence of α-amanitin, the specific inhibitor of RNA polymerase II [13]; RNA polymerase assay was performed as described by Viarengo et al. [3], but in the presence of 300 mM ammonium sulfate. Only nucleolar preparations containing no RNA polymerase II activity were employed.

Solubilization of protein kinase from nucleoli. Purified nucleoli were suspended in 75 mM NaCl, 50 mM Na_2SO_3, 24 mM EDTA, pH 7.5, 1 mM dithiothreitol and 50 μg/l of phenylmethanesulfonyl fluoride using a glass Potter-Elvehjem homogenizer fitted with a motor-driven Teflon pestle and were centrifuged at 4000 × *g* for 10 min. The pellet was washed once in the saline/EDTA solution and twice in 0.14 M NaCl/10 mM Tris · HCl, pH 7.5/50 μg/l of phenylmethanesulfonyl fluoride. The supernatants of the washing steps were combined and brought to 70% saturation by the addition of solid ammonium sulfate and centrifuged at 30 000 × *g* for 30 min; the pellet was resuspended in 10 mM Tris · HCl/1 mM dithiothreitol, 50 μg/l of phenylmethanesulfonyl fluoride and dialyzed against the same buffer overnight.

Assay of protein kinase activity. Unless described otherwise, protein kinase was assayed at 30° C in 0.1 ml of a reaction mixture containing 20 mM $MgCl_2$, 100 mM NaCl, 10 mM dithiothreitol, 0.25 M sucrose, 0.1 M Tris · HCl, pH 7.6, 8 mg/ml of casein, $2 \cdot 10^{-5}$ M [γ-^{32}P]ATP (1.0—1.5 Ci/mmole) and enzyme (15 μg of solubilized proteins). When indicated, 6 mM NaF was present in the reaction mixture. All reactions were carried out for a 10 min period and were terminated by pipetting 0.04 ml of the incubation mixture onto Whatman GF/C glass fiber filters and immediately placing them in ice-cold 10% trichloroacetic acid. The filters, washed by method B of Reimann et al. [14], were dried and counted for radioactivity in a Beckman LS 100 liquid scintillation spec-

trometer. Toluene containing 5% PPO was used as a scintillator.

Protein kinase activity was obtained from the difference between the ^{32}P incorporation into nucleolar proteins plus casein and ^{32}P incorporation into nucleolar proteins alone (endogenous phosphorylation). Protein kinase specific activity was defined as pmol of ^{32}P incorporated into casein/10 min per mg protein. Proteins were assayed by the method of Lowry et al. [15].

Chemicals. Vitamin-free casein (Merck, Darmstadt, G.F.R.) was suspended in water, heated at 100°C for 10 min, adjusted at pH 9.5 with NaOH and cooled. The pH was then lowered to 6.5. [γ-^{32}P]ATP was purchased from The Radiochemical Centre, Amersham, U.K. Cyclic AMP, calf thymus histones (type III-S lysine-rich; type VIII-S arginine-rich subgroup F_3), phosvitin, dithiothreitol, phenylmethanesulfonyl fluoride, protamine and aminophylline were purchased from Sigma, St. Louis, Miss., U.S.A..

Results

Requirements of the reaction and substrate specificity

The requirements of the reaction as well as the effects of the various components of the assay on the protein kinase solubilized from rat liver nucleoli are presented in Table I. It is apparent that Mg^{2+} is essential and that an appropriate concentration of NaCl (100 mM) is needed to achieve maximal rates of the reaction. On the other hand, the removal of NaF stimulates the protein kinase activity by about 30%. Furthermore, in the absence of casein the kinase activity is reduced to 8%, indicating that the solubilized enzyme preparation is relatively free of endogenous substrates. Finally, the reaction shows an absolute requirement for the enzyme excluding the nonenzymic binding of radioactivity to the acid-precipitable material.

The substrate specificity and the effect of cyclic AMP on the protein kinase are shown in Table II. It can be seen that the protein kinase solubilized from liver nucleoli phosphorylates casein more efficiently than phosvitin and displays an almost undetectable activity towards histones and protamine. No significant stimulation by cyclic AMP was detected.

TABLE I

REQUIREMENTS OF THE REACTION FOR PROTEIN KINASE ACTIVITY SOLUBILIZED FROM LIVER NUCLEOLI

The complete system was as described under Methods and contained 6 mM NaF. Protein kinase specific activity is defined as pmol of ^{32}P incorporated/mg protein per 10 min. The values represent the arithmetic mean of four experiments each performed in duplicate.

Reaction medium	Protein kinase specific activity	%
Complete system	2485	100
Omit $MgCl_2$	90	4
Omit NaCl	1640	66
Omit NaF	3260	131
Omit casein	250	8
Omit enzyme	0	0

TABLE II

SUBSTRATE SPECIFICITY AND EFFECT OF CYCLIC AMP ON PROTEIN KINASE SOLUBILIZED FROM LIVER NUCLEOLI

The specific activity of protein kinase using casein as substrate in the absence of cyclic AMP was set at 100%; specific activities with substrates and in presence of 2.5 μM cyclic AMP were compared to that value. 1.25 mM aminophylline was present in all cases. All protein substrates were adjusted to the concentration of 800 μg/ml. When histones were used as substrates the concentration of trichloroacetic acid in the washing solutions was raised to 25% (w/v) instead of the usual 10%. The values represent the arithmetic mean of four experiments each performed in duplicate.

Cyclic AMP	Casein	Phosvitin	Lysine-rich histone	Arginine-rich histone	Protamine
—	100	43	4	2.8	8
+	90	38	4	2.9	8

The pH profile for the phosphorylation of added casein is depicted in Fig. 1. It can be seen that the kinase activity increases significantly as the pH of the reaction medium increases from 6.4, with an optimum at 7.6. The kinase activity remains at high level between pH 7.8 and 8.4 and then declines rapidly. The apparent double maximum in the curve is not due to the use of different buffers. In fact, the same optima were found when Tris · HCl buffer was used in the range of pH from 6.8 to 8.6. Whether the shape of the pH profile is due to the presence of more than one protein kinase or whether it reflects a characteristic of a single enzyme cannot at present be assessed. However, increasing evi-

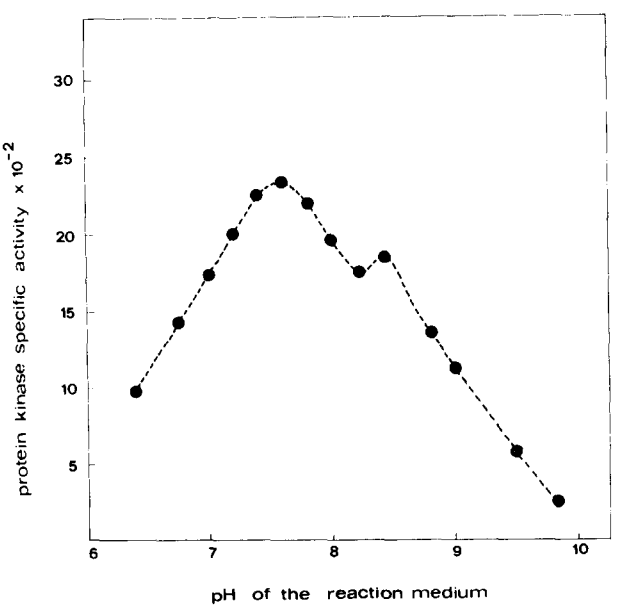

Fig. 1. Effect of pH on protein kinase solubilized from liver nucleoli. The pH values indicated on the abscissa refer to the final pH of the reaction medium measured at 30°C and were achieved by adding 100 mM imidazole · HCl (pH 6.45—7.2), 100 mM Tris · HCl (pH 7.2—8.2), or 100 mM $NaHCO_3/Na_2CO_3$ (pH 8.2—9.8) in the reaction medium. All the details were as described under Methods.

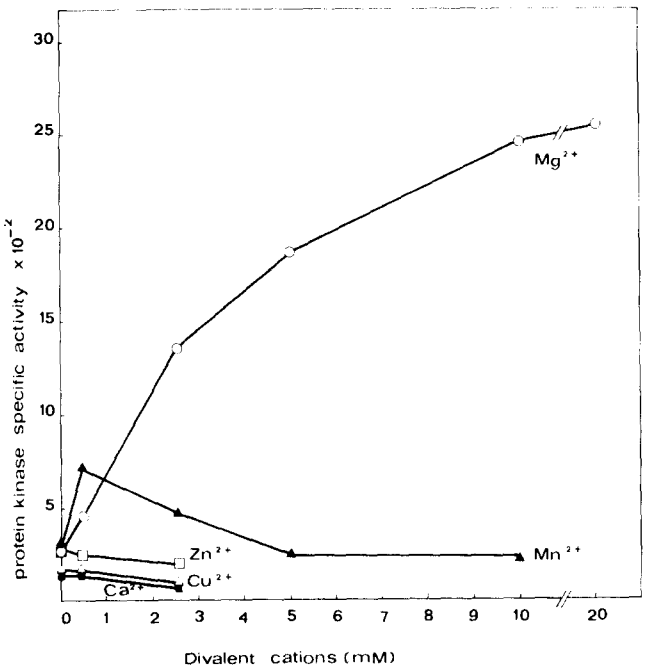

Fig. 2. Effect of divalent cations on protein kinase solubilized from liver nucleoli. Divalent cations were added to reaction mixture as chlorides. All the details were as described under Methods.

dence suggests that the protein kinase activity associated with isolated nucleoli is due to the presence of more than one enzyme [11,12].

As reported earlier, the kinase reaction shows an absolute requirement for Mg^{2+}. The results given in Fig. 2 demonstrate the optimal concentration of this

TABLE III

T_3 EFFECT ON NUCLEOLUS-ASSOCIATED PROTEIN KINASE ACTIVITY

The reaction mixture was as described under Methods and contained 6 mM NaF. In each experiment purified nucleoli (30 μg of protein) were incubated at 30°C for 10 min. The reaction was stopped by adding 0.5 ml of 5% trichloroacetic acid and the mixture was heated at 90—95°C for 15 min in order to hydrolyze RNA. The acid-insoluble material was collected on glass fiber filters and washed as reported in Methods. Protein kinase activity was obtained from the difference between the ^{32}P incorporation into nucleolar proteins + casein and ^{32}P incorporation into nucleolar proteins alone (endogenous phosphorylation). Data are expressed as pmol of ^{32}P incorporated/mg protein per 10 min. The values represent the mean ± S.E. of six experiments.

Treatment	Protein kinase activity	% increase	Endogenous phosphorylation	% increase
—	980 ± 60		289 ± 45	
T_3 12 h	1300 ± 80	+ 33	459 ± 50	+ 58
—	1130 ± 66		273 ± 48	
T_3 24 h	1538 ± 84	+ 36	415 ± 55	+ 52
—	1075 ± 104		354 ± 81	
T_3 48 h *	2550 ± 400	+137	885 ± 99	+150

* The rats received a second dose of T_3 24 h before killing.

TABLE IV

T_3 EFFECT ON PROTEIN KINASE SOLUBILIZED FROM LIVER NUCLEOLI

The reaction mixture was the same as described under Methods. Data are expressed as pmol of ^{32}P incorporated into casein/mg protein per 10 min. Corrections were applied for ^{32}P incorporation in the absence of casein. The values represent the mean ± S.E.

Treatment	No. of experiments	Protein kinase activity	% increase
—	3	2548 ± 120	
T_3 12 h	3	3193 ± 151	+ 25
—	4	2440 ± 117	
T_3 24 h	4	3637 ± 142	+ 49
—	3	2630 ± 139	
T_3 48 h *	3	5790 ± 485	+120

* The rats received a second dose of T_3 24 h before killing.

cation to be 10—20 mM. In addition, the effect of substituting divalent cations such as Mn^{2+}, Zn^{2+}, Cu^{2+} or Ca^{2+} for Mg^{2+} in the protein kinase assay was evaluated (Fig. 2). Mn^{2+} at low concentrations (up to 0.5 mM) can substitute advantageously Mg^{2+} in the stimulation of the kinase reaction whereas at concentrations above 2.5 mM it appears to have an inhibitory effect. Zn^{2+}, Cu^{2+} or Ca^{2+}, on the other hand, are essentially incapable of replacing Mg^{2+} in the stimulation of the kinase reaction.

Varying the casein or ATP concentrations in the kinase reaction gave the typical Michaelis-Menten-type kinetics. The apparent K_m values for casein and ATP, as determined from the intercepts of double-reciprocal plots of enzyme activity against substrate concentration, were 1.5 mg/ml and $1.5 \cdot 10^{-5}$ M, respectively.

T_3 effect on nucleolus-associated protein kinase activity

The data reported in Table III confirm earlier studies indicating that T_3 administration stimulates the in vitro phosphorylation of liver nucleolar proteins and demonstrate, in addition, that the hormone induces a net increase in the nucleolus-associated protein kinase activity after 12 h. An even larger increase was found after 24 and 48 h of treatment. As shown in Table IV, a hormone-mediated increase in protein kinase activity was also observed when solubilized enzyme preparations were employed. In addition, it has been shown that the administration of T_3 increases the V value of the reaction without affecting the apparent K_m values for casein and ATP of the solubilized enzyme (data not shown).

Discussion

The foregoing results indicate that T_3 administration to thyroidectomized rats induces a significant increase in liver nucleolar protein kinase activity. The enhanced level of kinase activity seems to reflect a higher concentration and not an increased catalytic efficiency of the enzyme, since the apparent K_m values for casein and ATP were not affected by T_3.

The physiological significance of the kinase(s) associated to liver nucleoli is at present little understood so that only hypotheses may be put forward in this regard. It is worth noting, however, that increases in nucleolar protein phosphorylation occur under conditions known to enhance the transcription of rRNA [16]. Therefore, the observed increase in nucleolar kinase activity as well as in the phosphorylation of nucleolar proteins may be a part of the mechanism of the T_3-induced stimulation of rRNA synthesis and RNA polymerase I activity [2,3]. In addition, as suggested by Olson et al. [17], several highly phosphorylated proteins are involved in the assembly and processing of preribosomal particles. According to this hypothesis, the increased phosphorylation of liver nucleolar proteins induced by T_3 administration may result in a stimulated maturation of preribosomal particles and, eventually, in the well known accumulation of newly formed ribosomes in the cytoplasm of liver cells [18].

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