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HISTONE KINASES AND CYCLIC AMP-BINDING CAPACITY OF NUCLEI OF HUMAN TONSILLAR LYMPHOCYTES

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SUMMARY

Isotonic salt extract of nuclei of human tonsillar lymphocytes contained about 60–65% of the total histone kinase activity (EC 2 7 1 37) of the cells The nuclear histone kinase activity was apparently cyclic AMP independent, although 20–25% of the total cyclic AMP-binding capacity of the cells was present in the nuclear extract

A cyclic AMP-dependent kinase (I), and an independent kinase (II), similar to those found previously in the hypotonic extract of the cells, were demonstrated in the nuclear extract by DEAE-cellulose chromatography In addition a second type of cyclic AMP independent histone kinase (III) was found On the basis of its catalytic properties, (kinetic parameters and the phosphorylation site of F2b histone), kinase III differed from kinase II, but it was similar to kinase I Kinase III may be identical with the catalytic subunit of the cyclic AMP dependent enzyme

A cyclic AMP-binding fraction accompanied by a negligible amount of kinase activity was also demonstrated in the nuclear extract by DEAE-cellulose chromatography

INTRODUCTION

The role of histone phosphorylation in the determination of chromosonal structure or in the regulation of some nuclear processes is the subject of speculation It is clear from studies on histone phosphorylation in living cells, that this type of histone modification may have various functions [1, 2] On the other hand, studies on histone kinases demonstrated that different histone phosphorylating enzymes do exist [3–5]

In spite of lack of any direct information, cyclic AMP dependent histone kinase is supposed to take part, at least to some extent, in the nuclear regulatory processes However, the nuclear histone kinase activity proved to be cyclic AMP independent [4, 6] The data presented may resolve this contradiction

MATERIALS AND METHODS

Chemicals

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) was purchased from

Sigma Chemical Co The F2b histone fraction was prepared according to the method of Johns [7] (Method I) [γ-^{32}P]ATP (200 Ci/mole) was produced by the photosynthetic method of Avron [8] Cyclic [8-^{3}H]AMP (27 5 Ci/mmole) was obtained from The Radiochemical Center Amersham DEAE-cellulose and Sephadex G-100 were the products of Koch-Light Laboratories and Pharmacia, respectively The other chemicals were of A R grade, obtained from Reanal (Budapest)

Isolation of lymphocytes

Tonsils from 4–10-year-old children were collected in ice, and used not later than 3–4 h after tonsillectomy In order to isolate lymphocytes, the tonsils were minced and the pieces stirred in 3 vol of Hanks' solution for 20 min The suspension was filtered through four layers of gauze, and centrifuged at 600 $\times$ g for 10 min The precipitate was washed with Hanks' solution All these procedures were carried out at 0 °C Lymphocytes were counted in a Burker chambre using Turk solution The isolation procedure yielded about 5 10^{8}–7 10^{8} cells per tonsils

Preparation of the hypotonic supernatant and of the nuclear extract

Washed lymphocytes from 15 pairs of tonsils were suspended in 60 ml of a hypotonic solution containing 0 04 M KCl, 0 002 M $MgCl_2$ and 0 01 M Tris–HCl buffer (pH 7 2) The suspension was stirred for 15 min and centrifuged (1700 $\times$ g) The supernatant thus obtained was called the hypotonic extract

The pellet from the hypotonic extraction procedure was suspended in 30 ml of KCl–$MgCl_2$–Tris–HCl buffer This suspension was layered on the top of 60 ml of KCl–$MgCl_2$–Tris–HCl buffer containing 30% sucrose, and centrifuged at 1700 $\times$ g for 15 min The nuclei obtained were resuspended in 15 ml of 0 9% NaCl–5 mM phosphate (pH 7 0), homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle, and centrifuged at 3500 $\times$ g for 20 min The homogenization was repeated with the pellet resuspended in 10 ml of the same solution The supernatants were combined and centrifuged at 32 000 $\times$ g for 60 min This procedure yielded the nuclear extract In some experiments, the combined supernatants were centrifuged for 60 min at 160 000 $\times$ g instead of 32 000 $\times$ g

Determination of histone kinase activity

The assay procedure was essentially the same as described by Kuo and Greengard [9] The standard reaction mixture contained 0 05 M sodium glycerophosphate (pH 6 5) 0 01 M $MgCl_2$, 0 002 M theophylline, 0 8 nmole [γ-^{32}P]ATP (200 Ci/mole) and 1 6 mg F2b histone in a final volume of 1 6 ml The reaction mixture was completed with 10^{-6} M cyclic AMP where indicated The reaction was carried out at 32 °C After 10 min preincubation, the reaction was started by the addition of the enzyme solution, and was stopped, generally after 10 min, with 4 ml of cold 40% trichloroacetic acid After centrifugation, the precipitate was dissolved in 0 5 ml of 1 0 M NaOH, and was reprecipitated by 4 0 ml of 20% trichloroacetic acid This procedure was repeated Finally the material dissolved in 0 5 ml of 1 0 M NaOH was washed into 5 ml of distilled water The radioactivity was measured in a liquid scintillation spectrometer, on the basis of the Cerenkov effect

The enzyme activity in some experiments was expressed in units One unit of histone kinase activity is the amount of enzyme, catalyzing the transfer of 1 0 pmole

phosphate per min under the standard conditions The cyclic AMP dependent kinase activity was defined as the difference in the enzyme activity measured in the presence and absence of 10^{-6} M cyclic AMP

Assay of cyclic AMP-binding

This assay was performed exactly as described by Gilman [10], using the property of the cyclic AMP receptor protein to bind to cellulose ester membrane filter (Sartorius 11307) The assay mixture contained 4 10^{-8} M cyclic [^{3}H]AMP (27 5 Ci/mmole) and 0 05 M acetate buffer (pH 4 0) Since this method is highly specific for cyclic AMP dependent protein kinase or cyclic AMP-binding protein [10], and saturating concentration of cyclic AMP was used, the concentration of cyclic AMP binding sites could be calculated directly from the amount of cyclic [^{3}H]AMP bound to the membrane filter (This amount was always less than 10% of the total amount of cyclic AMP in the assay mixture)

Miscellaneous

Preparation of histone kinase I and II from the hypotonic extract of lymphocytes from tonsils stored at −20 °C for several weeks, was carried out using DEAE-cellulose chromatography, as described [4] Histone kinase I was eluted from the column by 0 1 M phosphate, while histone kinase II was found in the 0 2 M eluate

The amount of protein was determined by the method of Lowry et al [11]

Phosphorylation of F2b histone by histone kinase III, was performed in 32 ml of the basal reaction mixture, for the analysis of ^{32}P-labelled histone peptides The reaction was stopped by the addition of 1 vol of cold 40% trichloroacetic acid After centrifugation, the precipitate was dissolved in 1 5 ml of 1 0 M NaOH, and reprecipitated with 30 ml of 20% trichloroacetic acid This procedure was repeated Finally the precipitate was dissolved in 1 5 ml of 1 0 M NaOH 1 5 ml of 1 0 M KH_2PO_4 and 7 0 ml of distilled water was added, than the solution was dialysed against distilled water The treatment of the phosphorylated sample and the fingerprint analysis was carried out exactly as in the previous experiments [4]

RESULTS

As reported [4], two different histone kinases have been separated by DEAE-cellulose chromatography from the hypotonic supernatant of human tonsillar lymphocytes One of these enzymes eluted from the DEAE-cellulose by 0 1 M phosphate (pH 7 0) was cyclic AMP dependent (histone kinase I), while the other, eluted by 0 2 M phosphate was independent on the cyclic nucleotide (histone kinase II) These two enzymes differed also in their kinetic properties and in the site of phosphorylation of the F2b histone fraction The crude extract of nuclei contained an apparently cyclic AMP independent histone kinase activity [4] To avoid the large endogenous substrate contamination, which hindered the purification of nuclear histone kinase previously, in the present investigations nuclei were desintegrated in isotonic buffer solution (5 mM phosphate buffer (pH 7 0) in 0 9% NaCl) and the suspension was centrifuged at 32 000 × *g* This procedure resulted in a nuclear extract, with a total histone kinase activity about two times higher than that of the hypotonic supernatant The nuclear histone kinase activity was apparently quite cyclic AMP independent, however, about

TABLE I

DISTRIBUTION OF HISTONE KINASE ACTIVITY AND CYCLIC AMP-BINDING CAPACITY BETWEEN THE HYPOTONIC SUPERNATANT AND THE NUCLEAR EXTRACT OF TONSILLAR LYMPHOCYTES

Extracts were prepared from 15 pairs of tonsils (1 8 10^{10} cells), and were dialysed against 0 005 M phosphate buffer (pH 7 0)

Fraction	Protein (mg/ml)	Histone kinase activity in the absence and presence of 10^{-6} M cyclic AMP			Cyclic AMP-binding	
		units/ml	units/ml	(units total)	pmole/ml	pmole/mg protein
Hypotonic supernatant (60 ml)	4 5	0 82	1 1	(66)	10 0	2 2
Nuclear extract (25 ml)	0 85	4 2	4 3	(107)	5 5	6 3

20–25% of the cyclic AMP-binding activity extracted from the cells was found in the nuclear extract Since the cyclic AMP-binding activity per mg protein was higher in the nuclear extract than in the hypotonic supernatant, the cyclic AMP-binding activity of the former could not be attributed to a contamination by the cytosol (Table I)

More than 90% of histone kinase activity of the nuclear extract was bound by DEAE-cellulose at pH 7 0, and the chromatography revealed histone kinase fractions similar to those separated from the hypotonic supernatant A cyclic AMP dependent histone kinase fraction appeared at 0 1 M phosphate while the cyclic AMP independent histone kinase II was eluted by 0 2 M phosphate (Fig 1)

However, the cyclic AMP activation of the nuclear histone kinase fraction eluted by 0 1 M phosphate was only 50–60%, while the cyclic AMP dependent histone kinase separated from the hypotonic extract of cells obtained from frozen tonsils was activated to 600–700% In addition, the histone kinase activity of the nuclear 0 1 M phosphate eluate was relatively high in the absence of cyclic AMP, hence this fraction seemed to contain a cyclic AMP independent histone kinase beside the cyclic AMP dependent enzyme

The cyclic AMP-binding activity eluted from the DEAE-cellulose did not coincide strictly with the cyclic AMP dependent histone kinase activity More than 50% of the cyclic AMP-binding activity, accompanied only by negligible amount of kinase I was found to follow the peak of this enzyme (Fig 1B)

The cyclic AMP independent component of the 0 1 M phosphate eluate was isolated from the 160 000 × *g* supernatant of the nuclear extract, by stepwise elution from DEAE-cellulose (Fig 2) The bulk of the cyclic AMP dependent kinase activity was absent from the 160 000 × *g* supernatant Instead of kinase I, a cyclic AMP independent histone kinase appeared in the 0 1 M phosphate eluate, practically without any cyclic AMP-binding activity (histone kinase III) Kinase II was eluted as usually by 0 2 M phosphate Their kinetic parameters being different, the two cyclic AMP independent histone kinases (II and III) can readily be distinguished on the basis of their behaviour in the presence of low and high substrate concentration (Fig 2B)

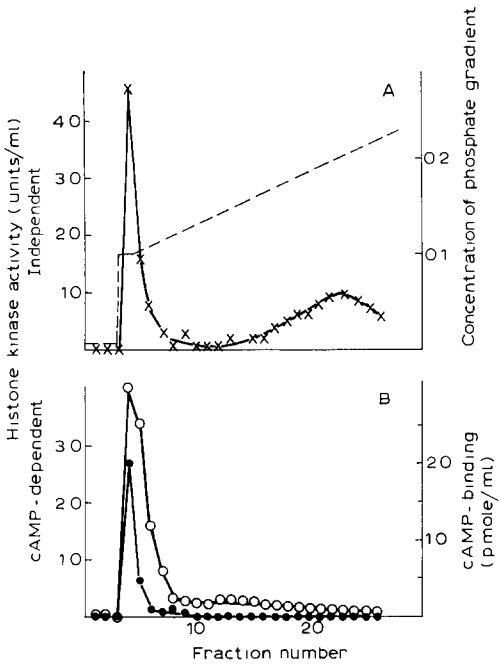

Fig 1 DEAE-cellulose chromatography of the nuclear extract (32 000 × *g* supernatant) 20 ml of nuclear extract (18 mg protein) dialysed against 0 005 M phosphate (pH 7 0) buffer, was put on the column (1 cm × 5 cm) equilibrated with the same buffer The column was washed with 30 ml of 0 005 M phosphate, and than a gradient was applied from 0 1 M to 0 3 M phosphate (pH 7 0) 5-ml fractions were collected Part A shows the histone kinase activity in the absence of cyclic AMP (×—×) The dotted line indicates the concentration of the eluent Part B shows the cyclic AMP dependent kinase activity (●—●), (expressed as the difference in the enzyme activity measured in the presence and absence of cyclic AMP) and the cyclic AMP-binding capacity (○—○)

The cyclic AMP binding activity which followed the peak of kinase I when gradient elution was used (Fig 1), was found right at the beginning of kinase II peak in the case of stepwise elution (Fig 2) Although this cyclic AMP-binding activity was accompanied by the traces of cyclic AMP dependent kinase, the cyclic AMP-binding activity was much higher than could be due to the enzyme alone (The rate of cyclic AMP-binding to cyclic AMP dependent kinase activity was about 6–8 times higher than this rate in a cyclic AMP dependent enzyme preparation)

The catalytic properties of histone kinase III were investigated in detail The substrate saturation curve of this enzyme with F2b (Fig 3), was similar to that of histone kinase I when measured in the presence of cyclic AMP, while kinase II showed a quite different substrate saturation [4] The apparent K_m for kinase III and I were 2 2 10^{-5} M and 1 5 10^{-5} M, respectively while the F2b concentration causing 50% of the maximal reaction velocity was found to be about 10^{-6} M in the case of kinase II (estimated on the basis of an anomalous saturation curve)

The pH optimum for kinase I and III were also similar, (between pH 6 0 and 6 5), while kinase II was more active at higher pH (Fig 4) Kinase II lost more than 50% of its activity when it was dialysed against 5 mM phosphate buffer (pH 7 0) while kinase I and III were stable under these circumstances

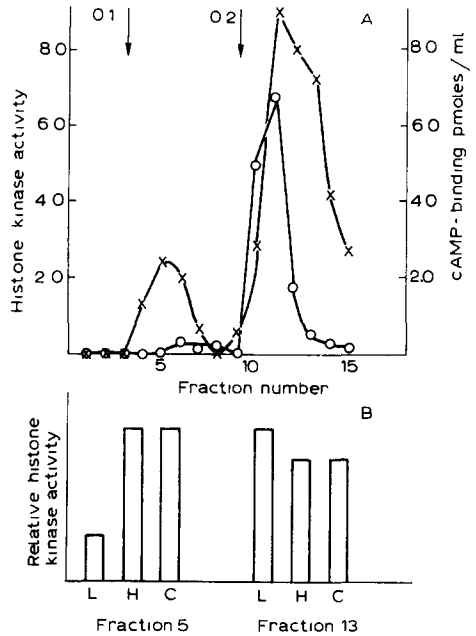

Fig 2 DEAE-cellulose chromatography of the 160 000 × *g* supernatant of the nuclear extract 20 ml of the 160 000 × *g* supernatant (20 mg protein) dialysed against 0 005 M phosphate (pH 7 0) was put on the column (1 cm × 5 cm) equilibrated with the same buffer The column was washed with 30 ml of 0 005 M phosphate, and than a stepwise elution was carried out using 30 ml of 0 1 M and 30 ml of 0 2 M phosphate (pH 7 0) 5-ml fractions were collected Part A shows the histone kinase activity (units/ml) in the absence of cyclic AMP (×—×) and the cyclic AMP binding capacity (○—○) Part B presents the relative kinase activities measured in the presence of low and high substrate concentration of the 5 fraction (kinase III) and of the 13 fraction (kinase II) L in the presence of 0 1 mg/mlF2b histone, H 1 0 mg/ml F2b and C 1 0 mg/ml F2b + cyclic AMP

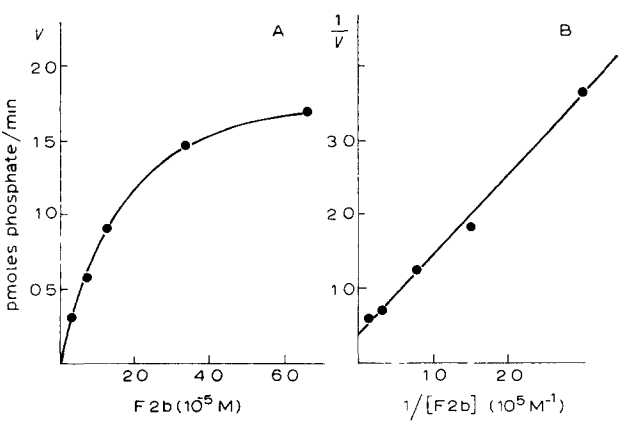

Fig 3 F2b histone saturation curve of kinase III The reaction was carried out in the standard reaction mixture, containing 0 08 mg "enzyme" protein

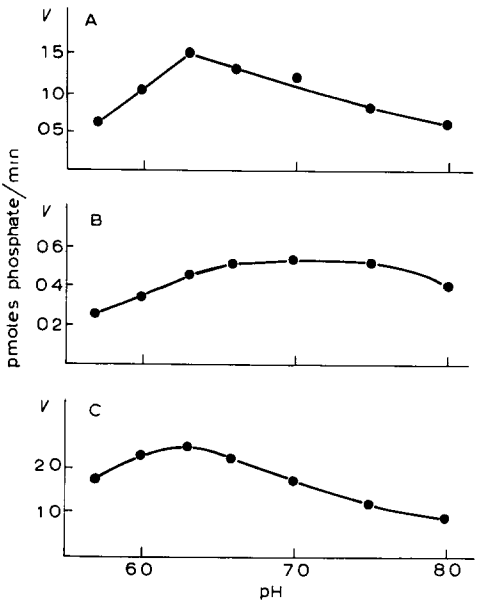

Fig 4 The effect of pH on the activities of the three histone kinases The activity of kinase I was measured in the presence of cyclic AMP (Part A), while those of kinase II (Part B) and kinase III (Part C) were measured in the absence of the cyclic nucleotide The standard reaction mixture contained 0 05 M phosphate instead of β-glycerophosphate "Enzyme" protein per sample was 0 1 mg, 0 06 mg and 0 8 mg for A, B and C, respectively

Previous results obtained from the fingerprint analyses of ^{32}P-phosphorylated F2b histone, showed difference between the action of kinase I and II Kinase I was found to phosphorylate preferentially one peptide fragment of the F2b fraction, while four different peptide fragments were phosphorylated nearly equally by kinase II [4] The fingerprint analysis of F2b histone phosphorylated by kinase III gave similar result as was found characteristic for the action of kinase I About 90% of the radioactivity was localized in the same single peptide fragment, indicating that histone kinase III phosphorylated the same site of F2b as the cyclic AMP dependent enzyme

DISCUSSION

In our previous investigations the major part of nuclear histone kinase activity was not bound by DEAE-cellulose at pH 7 0, presumably because the large amount of endogenous histone present in the 5 mM phosphate extract of nuclei hindered the binding In the present experiments nuclei were desintegrated in isotonic buffer, and the histone kinase activity of this isotonic nuclear extract was bound by DEAE-cellulose We cannot exclude the possibility that some histone kinase activity was not extracted from the nuclear material by this procedure, but our results demonstrated that nuclei of human tonsillar lymphocytes contained the same two histone kinase (I and II) as the cytosol

A third type of histone kinase (III) was also separated from the nuclear extract, however this enzyme had quite similar properties as the cyclic AMP dependent enzyme, except, that it was not activated by cyclic AMP and had no cyclic AMP binding

activity The cyclic AMP dependent histone kinase is known to contain a catalytic subunit and a regulator subunit, which dissociate from each other on the effect of cyclic AMP [12–15] Histone kinase III is probably identical with the catalytic subunit of histone kinase I, hence our data suggest that a part of the cyclic AMP independent histone activity of the nuclei may derive from the cyclic AMP dependent enzyme

The presence of the free catalytic subunit in the nuclei can be explained by the high cyclic AMP level of tonsillar lymphocytes as compared to other tissues The high cyclic AMP content of these cells may be related to the immunological function of lymphocytes [16] Cells isolated from fresh tonsils contain about 60 pmoles cyclic AMP per 10^7 cells (Faragó, A , to be published) This cyclic AMP level has to cause the dissociation of a part of the cyclic AMP dependent enzyme molecules A somewhat similar phenomenon was reported by Cross and Ord [17], who found the cyclic AMP dependency of histone kinase from pig blood lymphocytes to decrease rapidly (within one hour), when the lymphocytes were treated with phytohemogglutinin

The chromatographic profile of the nuclear extract indicated a significant amount of cyclic AMP-binding activity without, or with strongly reduced kinase activity Among the possible explanations of this cyclic AMP-binding activity, the most probable seems to be, that the free regulator subunit of the cyclic AMP dependent enzyme is also present in the nuclear extract If kinase III is identical with the free catalytic subunit, the existence of the free regulator subunit is to be expected In addition, as it was shown by Sephadex gel filtration, two kinds of molecules having cyclic AMP-binding activity were present even in the hypotonic extract of human tonsillar lymphocytes one with a molecular weight of about 95 000, and an other of about 55 000 The former was presumably the cyclic AMP dependent enzyme, while the latter might be the free regulator, since the molecular weight of the cyclic AMP dependent histone kinase of human blood lymphocytes, and that of the catalytic subunit of this enzyme is about 90 000 and 40 000, respectively [18]

The number of cyclic AMP binding sites per tonsillar cells is in the order of 10^4, and a considerable part of these sites is inside the nucleus The question arises, whether the only function of these cyclic AMP receptor molecules is to inhibit the catalytic activity of the histone kinase or perhaps of some other enzyme It is well known that in prokaryotic cells a cyclic AMP-receptor complex stimulates the synthesis of lac messenger RNA, and this effect is not restricted to one operon, but a series of adaptive enzyme systems are influenced by the cyclic AMP-receptor complex [19] Regulation of nucleic acid synthesis of eukaryotic cells differs substantially from that of prokaryotes, and in eukaryotes one effect of cyclic AMP on the nuclear processes, i e the enhancement of histone phosphorylation has been clarified However, concommitant with this function, cyclic AMP might have also some other role in the regulation of nuclear processes [20], which involves the cyclic AMP–receptor complex dissociated from the catalytic subunit

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