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CHROMATIN-ASSOCIATED PROTEIN KINASES SPECIFIC FOR ACIDIC PROTEINS

HANS STAHL and ROLF KNIPPERS

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.)

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Summary

Protein kinases (EC 2.7.1.37) were eluted by 0.4 M NaCl from chromatin of several mammalian cell types. The enzymes were partially purified by ion-exchange chromatography, DNA-cellulose columns and sucrose gradient centrifugation. At least five different enzymes could be distinguished by their biochemical properties and their substrate specificities.

Three of the enzyme activities tested phosphorylate different sets of histones, while two enzymes phosphorylate acidic nonhistone chromatin proteins or artificial substrates like casein and phosvitin. The two nonhistone protein kinases have slightly different pH and salt optima. They sediment through sucrose gradients with approx. 4 S and approx. 8 S, respectively. These enzymes are further characterized by their different substrate specificity, since they phosphorylate different, though partially overlapping sets of nonhistone chromatin proteins.

Enzymes with these properties were detected in chromatin from mouse ascites cells, bovine lymphocytes, African green monkey kidney cells and a human SV40 transformed cell line.

Introduction

The phenomenon of nuclear protein phosphorylation has been a field of intensive study since the first discovery of an unexpectedly high content of phosphorylated serine and threonine residues in acidic chromatin proteins [1—3].

The spectrum of acidic nuclear phosphoproteins varies from cell type to cell type and changes during normal growth and development, during stimulation by hormones or during malignant transformation (for review, see Refs. 4—6).

The phosphorylation of acidic nuclear proteins is often considered to play an important role in the activation of chromatin for transcription. This assumption is based on the correlation between enhanced RNA synthesis and protein phosphorylation. For instance, when resting lymphocytes are stimulated by concanavalin A or other mitogens to proceed to a metabolically more active state, the rates of nonhistone chromatin protein phosphorylation and of [^{3}H]-uridine incorporation into RNA increase during the same time period, 4—8 h, after addition of the lectin [7—9]. (An increase in histone phosphorylation occurs much later, beginning at the time of the DNA replication phase [7,8].)

To enable an understanding of the potentially important protein phosphorylation reaction, we have examined whether protein kinases associated with chromatin can be found which specifically catalyze the phosphorylation of acidic nuclear protein and if so, whether they can be distinguished from the better known histoné-specific protein kinases (for reviews, see Refs. 10 and 11). A number of publications have reported the presence of protein kinase(s) among the nonhistone chromatin proteins of the cell nucleus [6,10,12]. These enzyme activities can be assayed by incubation of salt-released chromatin proteins or of isolated 'intact' chromatin with [γ-^{32}P]ATP. Under these conditions the chromatin-bound enzymes phosphorylate at least some of their potential endogenous nonhistone substrates [13].

In this communication, we present a biochemical analysis of two major chromatin protein kinases specific for nonhistone chromatin proteins. Enzymes with similar properties are found associated with chromatin from four different mammalian cell types.

Material and Methods

Cells

Ehrlich ascites tumor cells were injected intraperitoneally into Balb/c mice. The cells were harvested 8—10 days after inoculation. The cells were stored at —70°C until use. Lymphocytes were prepared from bovine lymph nodes and cultivated as described [14]. Lymphocytes were stimulated to proliferate by 5 μg/ml concanavalin A. The African green monkey cell line as well as the human SV40 transformed SV80 cells were grown in monolayer cultures in Dulbecco-modified Eagle's medium (GIBCO) supplemented with 10% calf serum (Flow Laboratories) at 37°C in a 5% CO_2 atmosphere.

Preparation of chromatin

Chromatin was prepared according to Hancock [15] with the minor modifications described previously [13]. The chromatin preparation was washed three times with buffer A (50 mM Tris-HCl (pH 7.6)/5 mM EDTA/10 mM 2-mercaptoethanol/10% glycerol) and finally centrifuged through 10% sucrose in buffer A.

Preparation of chromatin-bound protein kinases

The washed chromatin was resuspended in buffer A containing 0.4 M NaCl, and kept on ice for 20—30 min. The insoluble chromatin was centrifuged at 10 000 rev./min at 0°C for 10 min in a Sorvall centrifuge. The supernatant

proteins were dialyzed in an ice bath for 12—16 h against buffer A. The precipitate which had formed during dialysis was removed by centrifugation (5 min at 2000 × *g*). The supernatant was applied to a A25 DEAE-Sephadex column which had been equilibrated with buffer A. The column was washed with buffer A and then eluted with a linear 0—0.45 M NaCl gradient (in buffer A). For further purification, fractions containing enzyme activity were precipitated in 60—65% $(NH_4)_2SO_4$, dialyzed and centrifuged through sucrose gradients as described below. In several experiments, fractions containing protein kinase activity were passed over DNA-cellulose columns [16]. As described below, some of the protein kinase activities are bound to DNA-cellulose columns while others pass through. The DNA-bound enzymes are eluted by 0.05 and by 0.5 M NaCl. All column chromatography steps are performed in glycerol-containing buffers at —5°C. We estimate a 200-fold purification over the crude preparation of salt-released nonhistone chromatin proteins.

Protein kinase activity was assayed in 0.1 ml of 5—10 mM sodium phosphate (pH 7.0)/10 mM $MgCl_2$/10 mM 2-mercaptoethanol. To this buffer were added 10 μM [γ-^{32}P]ATP (spec. act. 1000—2000 cpm · pmol^{-1}) and 5—10 μl fractions of the enzyme preparation to be tested. When a histone-specific protein kinase was to be assayed, 20 μg commercial calf thymus histones (Sigma Corp.) were added to the reaction mixture. Protein kinases that prefer nonhistone chromatin proteins as phosphate-accepting substrates are conveniently assayed using 20 μg phosvitin (Sigma Corp.) as acceptor protein. In some experiments, 20 μg casein (Sigma Corp.) was used as the phosphate-accepting substrate. We will show below that the use of these substrates allows a clear distinction between the histone specific and nonhistone specific protein kinase activities in chromatin. The incubation was carried out at 37°C usually for 30 min. At the end of the incubation period, the [^{32}P]phosphate transferred to protein was determined as the radioactivity which precipitates in 20% trichloroacetic acid. The preparation of the precipitates for radioactivity counting has been described [13].

Preparation of nonhistone chromatin proteins

A chromatin preparation was washed with 0.4 M NaCl to remove the endogenous protein kinase activity (see preceding paragraph). To this preparation was added an equal volume of 4 M NaCl and 1/10 volume of 1 M Na_2HSO_3. After several h with slow stirring in the cold, the preparation was centrifuged for 20—30 h at 100 000 × *g*. The supernatant was mixed with Bio Rex 70, previously equilibrated with 2 M NaCl in 0.1 M sodium phosphate, pH 7.0. The suspension was then dialyzed vs. several changes of 0.1 M sodium phosphate (pH 7.0)/0.01 M 2-mercaptoethanol [17]. After dialysis the Bio Rex 70 was poured into a column and washed with 0.1 M sodium phosphate buffer. Some nonhistones did not bind to Bio Rex 70 and others were eluted with 0.5 M NaCl in sodium phosphate while histones remained on the column (and can be eluted by 2 M NaCl). The two fractions of nonhistones were combined, dialyzed against water and lyophilized. This fraction of nonhistones contained most of the acidic proteins remaining on chromatin at a 0.4 M NaCl wash.

Other techniques

Polyacrylamide gel electrophoresis was performed essentially as described in Ref. 18. To determine the radioactivity, the gels were dried and exposed for the desired length of time on a Kodak X-ray film.

The conditions for sucrose gradient centrifugation will be given below.

Results

Chromatographic separation of chromatin-associated protein kinases from mouse ascites cells

We present in Fig. 1 the distribution of chromatin-associated protein kinase activities in a DEAE-Sephadex chromatogram. The following points should be noted: (i) the elution pattern of enzymatic activities using histones as phosphate-accepting substrates is different from that of enzymatic activities using

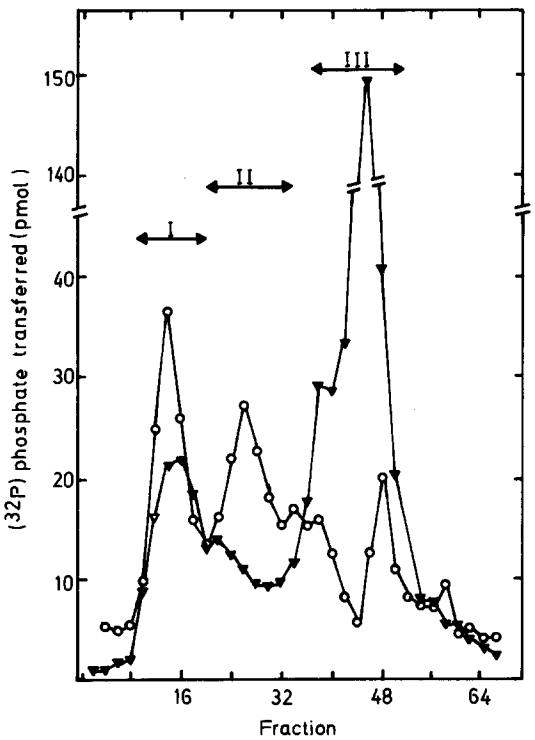

Fig. 1. Chromatography of chromatin-associated protein kinases from mouse ascites cells. 25 g mouse ascites cells were washed three times with 100 ml each of 1 mM potassium phosphate buffer, pH 7.5, in 5% sucrose. Chromatin was prepared according to Hancock's procedure [15]. After washing in buffer A, the chromatin preparation was resuspended in buffer A, containing 0.4 M NaCl. The solubilized proteins [12] were dialyzed, precipitates were removed by centrifugation and the supernatant was added to a A25 DEAE-Sephadex column (2 X 20 cm). Elution was performed with a linear gradient 0-0.45 M NaCl in a total volume of 300 ml buffer A. All operations were carried out at approx. —5°C in a Colora box. The protein kinase activity in the indicated fractions was determined using either calf thymus histones (o———o) or phosvitin (▾———▾) as the phosphate-accepting substrate. The amount of phosphate transferred to these substrates was estimated from the known specific radioactivity of the [γ-^{32}P]ATP used. The horizontal arrows indicate the pooled fractions which were precipitated by $(NH_4)_2SO_4$ and used for further purification by sucrose gradient centrifugation.

phosvitin as a substrate; (ii) there are four main peaks of histone-specific protein kinase activity and two major activity peaks which are most active on phosvitin. A part (approx. 10% of the recovered histone specific enzymatic activity) does not bind to DEAE-Sephadex and is detected in the flow-through.

The chromatographic profile in Fig. 1 is highly reproducible among different ascites cell chromatin preparations. Similar elution patterns are also seen when the chromatin-associated protein kinases from other cell types are chromatographed on A25 Sephadex columns (see below).

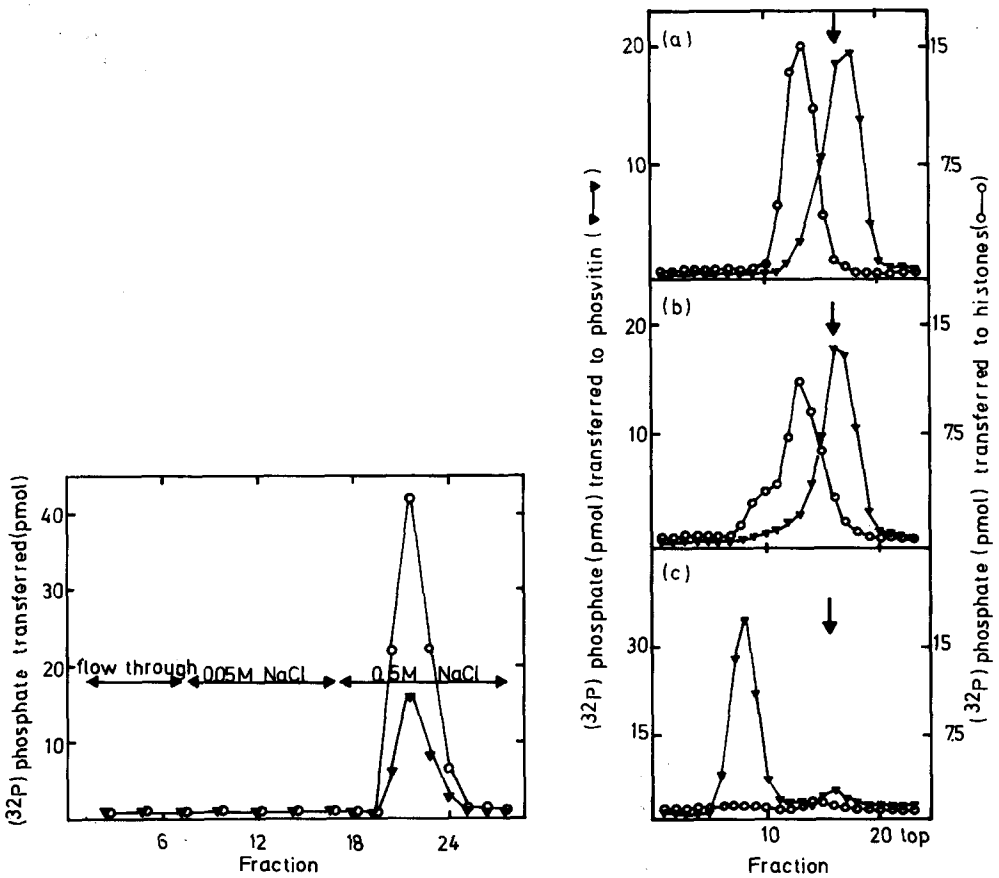

Fig. 2. Binding of protein kinases to DNA-cellulose. A mixture of the protein kinase samples from a A25 DEAE-Sephadex column (see Fig. 1) was dialyzed against buffer A and applied to a DNA-cellulose column (3 X 6 cm; ca. 0.8 mg heat denatured calf thymus DNA bound to 1 ml cellulose suspension). The column was washed first with 50 ml buffer A followed by 100 ml 0.05 M NaCl (in buffer A). The absorbed protein kinase activity was then eluted with 0.5 M NaCl. The protein kinase assay was carried out in the presence of histones (o———o) and of phosvitin (▼———▼) as phosphate-accepting substrates.

Fig. 3. Centrifugation analysis of chromatin-associated protein kinases. Samples as defined in Fig. 1 were centrifuged through linear 20%—5% sucrose gradients, containing 0.4 M NaCl in buffer A (plus 10% glycerol and 1 mM phenylmethylsulfonyl fluoride). The Beckman SW41 rotor was used at 38 000 rev./min and 0°C for 60 h. Bovine hemoglobin (indicated by the vertical arrow) served as sedimentation marker (4.2 S) and was identified by its characteristic absorption at 425 nm. The gradients were collected from the bottom. Protein kinase activity was tested in each fraction using histones (o———o) or phosvitin (▼———▼) as phosphate-accepting substrate. a, Sample I; b, sample II; and c, sample III of the A25 DEAE-Sephadex column chromatogram as shown in Fig. 1.

It has been shown before that most of the histone-specific chromatin-associated protein kinase activity from ouse ascites cells firmly binds to DNA-cellulose (or DNA-agarose) columns [19]. An experiment is presented in Fig. 2 to demonstrate that the nonhistone protein kinases also attach to DNA-cellulose columns. The DNA bound enzymes are eluted from the column by 0.5 M NaCl.

Characterization by zone velocity sedimentation

For a further characterization, the fractions indicated in Fig. 1 were combined, precipitated by $(NH_4)_2SO_4$, dialyzed and centrifuged through linear sucrose gradients using bovine hemoglobin as a sedimentation marker. The results are shown in Fig. 3. It can again be seen that in most cases histone-specific protein kinase activities are well separated from those using phosvitin as the preferred phosphate-accepting substrate.

Histone-specific protein kinases

The histone-specific protein kinases detected in the experiments of Fig. 1 and Fig. 3 have been described before. To give a complete description of the data presented in this paper we summarize briefly our analysis of the recovered histone-specific enzymes in Table I. The classification given in Table I is based mainly on the results of a gel electrophoretic analysis of the phosphorylated histones (Fig. 4).

Biochemical properties of nonhistone protein kinases

We find by sucrose gradient analysis two forms of nonhistone protein kinase, one sedimenting with approx. 4 S (Fig. 3a and b), and another one with approx. 8 S (relative to the hemoglobin marker) (Fig. 3c). To test for the specificity of these activities the enzyme maxima were incubated in the presence of [γ-^{32}P]ATP with the nonhistone chromatin preparation described under Material and Methods. The phosphorylated proteins were analyzed by polyacrylamide gel electrophoresis as shown in Fig. 5. Since the function of the detectable nonhistones is not known, the data of Fig. 5 are presented only to demonstrate clearly that the 4 S protein kinase has a substrate specificity which distinguishes this enzyme from the 8 S nonhistone protein kinase. We take this different (though overlapping) substrate specificity to indicate that there are at least two protein kinases in ascites cell chromatin which prefer nonhistones as phosphate-accepting substrates. The activities can be distinguished by their sedimentation coefficient ('4 S kinase', '8 S kinase') and by their specificity as far as the phosphate-accepting nonhistone substrates are concerned. We now

TABLE I

HISTONE-SPECIFIC PROTEIN KINASE ACTIVITIES RELEASED FROM ASCITES CELL CHROMATIN

Specificity	Sucrose gradient analysis	Ref.
H1	6 S (Fig. 3a; 3b)	20
H2b, H3, H4	7—8 S (Fig. 3b, leading shoulder)	21
H2b	not tested (flow-through of the DEAE-Sephadex column)	22

present some additional data as a contribution to a biochemical characterization.

Both the 4 S kinase and 8 S kinase, require magnesium salts for activity. No phosphate transfer is observed when Mg^{2+} is replaced by Ca^{2+} or Mn^{2+}. In fact, the addition of 2—6 mM $CaCl_2$ is strongly inhibitory when added to a reaction mixture with 5 mM $MgCl_2$ (which, alone, is optimal for the two nonhistone kinases tested) (Table II). Low concentrations of spermidine partially inhibit the 4 S kinases while the 8 S kinase is not affected by the spermidine concentrations used (Table II). The pH optima in sodium phosphate buffer are 6.0—6.5 for the 4 S kinase and approx. 7.0—7.5 for the 8 S kinase, respectively. The 4 S kinase is most active at 20—40 mM NaCl, the 8 S kinase at 50—100 mM. At 200 mM NaCl, both enzymes have 5—10% of their optimal activity. None of the enzymes tested responds to cyclic-AMP or cyclic-GMP in concentrations between 10^{-7}—10^{-5} M (see also Ref. 13).

All experiments described in the preceding paragraph were performed using phosvitin as an artificial phosphate-accepting substrate. Identical data were obtained with casein (not shown). (Several other proteins, including protamin sulfate, egg white lysozyme, human γ-globulin, were tested. They are not phosphorylated by the 4 S or 8 S nonhistone kinase.)

Chromatin-associated nonhistone protein kinases in other mammalian cell types

In an attempt to demonstrate the presence of the chromatin-bound nonhistone protein kinases described above in other mammalian cell systems we chromatographed on DEAE-Sephadex protein preparations from 0.4 M NaCl washes of chromatin prepared from concanavalin A activated bovine lymphocytes, African green monkey kidney cells and human SV40 transformed fibroblasts. All elution profiles are comparable to that shown in Fig. 1 for ascites cell chromatin. There was always a smaller peak I, eluting at approx. 0.1 M NaCl, and a larger peak II, eluting at approx. 0.3 M NaCl, of nonhistone kinase activity (data not shown).

As in the material from ascites cell chromatin, the peak I activity sediments through sucrose gradients with about 4 S and the peak II activity with about 8 S. The biochemical differences noted above for the corresponding ascites cell nonhistone protein kinases are also found for the enzymes from other sources. We present in Fig. 6, one experiment to show that the biochemical similarities of homologous enzymes from various sources are also apparent when they are

TABLE II

EFFECT OF Ca^{2+} AND SPERMIDINE ON NON-HISTONE PROTEIN KINASES

Divalent cations (mM)	(pmol ^{32}P-phosphate, transferred)	
	4 S Kinase	8 S Kinase
10 $MgCl_2$ plus:	19.5	19.0
2 $CaCl_2$	3.1	2.1
4 $CaCl_2$	1.2	0.9
6 $CaCl_2$	0.8	0.6
3 spermidine	11.5	19.6

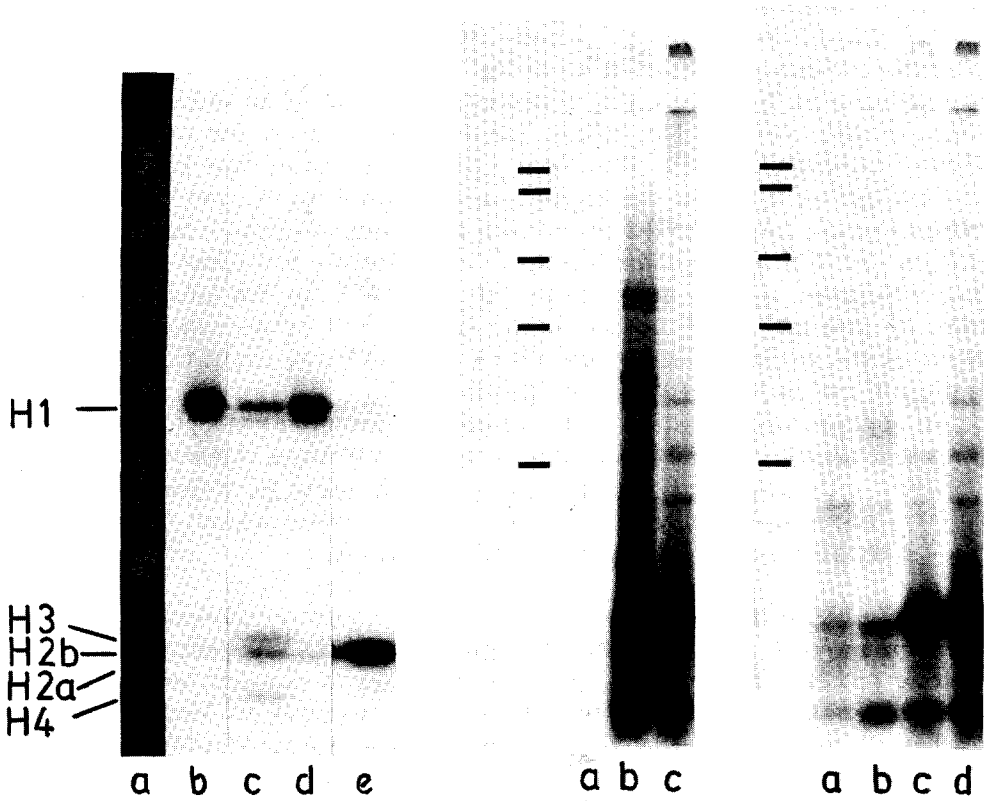

Fig. 4. (Left) Substrate specificity of histone protein kinases. Histone-specific protein kinase activities from the sucrose gradients shown in Fig. 3 were incubated with commercial calf thymus histones in the presence of [$\gamma - {}^{32}P$]ATP. After 30 min at 37°C, an aliquot of the reaction mixture was added to the sodium dodecul sulfate (SDS) containing sample buffer for electrophoresis [18]. After boiling, the samples were applied to a 15% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue, dried and used for autoradiography. a, Stained gel. Source of enzyme used: b, fraction 12 of the gradient in Fig. 3a; c, fraction 9 of the gradient in Fig. 3b; d, fraction 13 of the gradient in Fig. 3b; e, flow-through of the DEAE-Sephadex column (see Fig. 1).

Fig. 5. (Centre) Substrate specificity of the nonhistone protein kinases. The 4 S kinase (b) (fraction 16 of the gradient shown in Fig. 3a) and the 8 S kinase (c) (fraction 8 of the gradient of Fig. 3c) were incubated each under standard conditions in the presence of [γ-^{32}P]ATP with about 20 μg of the nonhistone chromatin protein preparation described under Material and Methods. To demonstrate the absence of endogenous protein kinase activity in this preparation, a 20 μg sample of the nonhistones was incubated with [γ-^{32}P]ATP without added protein kinase (a). After incubation the samples were prepared for electrophoresis in SDS-containing buffer [18] on a 12% polyacrylamide slab gel. After electrophoresis, the gel was stained, dried and used for autoradiography. Control experiments have shown that all radioactive bands correspond to phosphoproteins: (i) the bands cannot be detected after incubation with highly purified trypsin or pronase prior to electrophoresis; (ii) more than 95% of the associated radioactivity was alkali labile as expected for phosphoserine and phosphothreonine (data not shown). The horizontal bars indicate the position of electrophoretic markers which were run on the same gel (from upper to lower): β-galactosidase (M_r, 132 000), phosphorylase A (M_r, 94 000), glucose-6-phosphate kinase (M_r, 81 000), bovine serum albumin (M_r, 68 000), glutamatedehydrogenase (M_r, 53 000).

Fig. 6. (Right) Substrate specificity of 8 S kinases from different mammalian cells. The 8 S kinase from SV80 cells (a), from lymphocytes (b), from CV-1 cells (c) and from mouse ascites cells (d) were prepared by DEAE-cellulose chromatography and sucrose gradient centrifugation as described above. The enzymes were each incubated with [γ-^{32}P]ATP in the presence of the nonhistone chromatin preparation (see: Materials and Methods). After incubation, the samples were processed for electrophoresis [18] on 12% polyacrylamide slab gels. The dried gels were used for autoradiography. The horizontal bars represent the same electrophoresis markers as indicated under Fig. 5.

used to phosphorylate a given preparation of nonhistone chromatin proteins. The autoradiogram shown in Fig. 6 demonstrates that all 8 S kinases have some common substrates among the nonhistone chromosomal proteins tested. There are, however, some differences as indicated by several radioactive bands which appear on one but not on other lanes of the slab gel.

Discussion

Several types of chromatin-associated histone-specific protein kinase are known. Each type is characterized by the set of histones which are modified by phosphorylation after incubation of all five histones in the presence of ATP. In the DEAE-Sephadex chromatograms of salt-eluted chromatin proteins we could distinguish at least three different types of histone-specific protein kinase (Table I). This group of enzymes is clearly different from a second group of protein kinases which do not use the basic histones as phosphate-accepting substrates but prefer more acidic protein substrates. This group of protein kinases can be conveniently assayed by using phosvitin or casein as substrates. This group of nonhistone-specific protein kinases consists of at least two species which are distinguished by their chromatographic behaviour on DEAE-Sephadex columns (Fig. 1), by their sedimentation properties (Fig. 3) and their substrate specificity when assayed with a mixture of nonhistone chromatin proteins (Fig. 5). Both types of nonhistone protein kinase bind strongly to DNA-cellulose (Fig. 2) or to DNA-Sepharose (data not shown) columns. This behaviour may explain why the nonhistone kinases are detected in close association with chromatin. This latter remark deserves a comment, however.

The major part of cellular protein kinase activity corresponds to the two well known types of 'cytoplasmic' cyclic-AMP-dependent protein kinase [24] and is removed during the preparation of chromatin. After the last step in our preparation procedure (i.e. after centrifugation of the chromatin preparation through a sucrose column in buffer A protein kinase activity was no longer detected in the supernatant but a substantial fraction of enzymatic activity stayed on the chromatin pellet [13]. The protein kinase activity which we have described is thus firmly associated with chromatin. This does not necessarily mean that enzymes, similar or identical to those remaining on chromatin, may not also be found in a soluble form in the cytoplasm or in the nucleoplasm. In fact, it is quite possible that there may be an equilibrium between soluble and chromatin-bound nonhistone proteins. This notion is in agreement with the observations of Comings and Harris [24], who conclude that practically all nonhistone proteins in chromatin have a soluble 'cytoplasmic' counterpart. What we have described is that fraction of chromatin protein kinase activity which remains part of the nucleoprotein complex after extensive washing in our standard Tris-HCl buffer. A detectable contamination of these protein kinase fractions by the typical cyclic-AMP-dependent cytoplasmic protein kinase has been excluded [19]. It has also been shown before that 0.4 M NaCl releases more than 90% of all protein kinase activity from the insoluble nucleoprotein complex [13]. It is not known, however, whether nonhistone protein kinase activities of the type described in this communication occur in the soluble fraction of the cell. Their amount in the cytoplasm can certainly not be

large since more than 95% of all soluble protein kinase activity passes through DNA-cellulose columns (unpublished observations).

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