ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 189, No. 2, August, pp. 372–381, 1978

Protein Kinases in Human Renal Cell Carcinoma and Renal Cortex

A Comparison of Isozyme Distribution and of Responsiveness to Adenosine 3′:5′-Cyclic Monophosphate[1]

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Received November 23, 1977; revised February 8, 1978

Two major isozyme forms of cyclic AMP-dependent protein kinase (termed protein kinase I and II according to their order of elution from DEAE-cellulose) were resolved by DEAE-cellulose chromatography of extracts from human renal cortex and renal cell carcinoma. The ratio between protein kinase I and protein kinase II in carcinoma extracts was about twice that in extracts of renal cortex. The total soluble cyclic AMP-dependent protein kinase activity was similar in extracts from the normal and malignant tissue. Protein kinase isozymes prepared from renal cortex or carcinoma were highly dependent on cyclic AMP for activity under appropriate assay conditions, were activated to the same degree by various concentrations of cyclic AMP, and had similar affinity for the nucleotide, indicating that the mechanism for regulation of protein kinase activity by cyclic AMP was intact for the tumor kinases. The kinetics of endogenous phosphorylation of protein kinase II was similar for enzyme derived from normal or malignant tissue.

The cyclic AMP-dependent protein kinases (ATP-protein phosphotransferase, EC 2.7.1.37), whose activity is regulated by the dissociation of the holoenzyme into regulatory and active catalytic moieties in the presence of cyclic AMP (1), exist in mammalian tissues as two main isozyme forms termed protein kinase I and protein kinase II according to their order of elution from DEAE-cellulose by salt gradients (2).

Recent studies describing either a reduced kinase activity (3, 4), an altered isozyme-distribution (5), or lowered responsiveness to cyclic AMP (3, 4, 6) in neoplastic cell lines or experimental tumors, and correlation between the responsiveness of kinases to cyclic AMP and the sensitivity of tumor growth to cyclic AMP or its analogues (7, 8) point to a potential coupling between neoplastic growth and altered protein kinase also in human tumors. The aim of the present study was to compare the isozyme pattern of cyclic AMP-dependent protein kinases and the regulatory properties of those enzymes from a human carcinoma and its normal counterpart. Renal cell carcinoma was chosen because nontumorous renal cortical tissue could be obtained from the tumor-involved kidney upon its therapeutic removal, and because the dominating cell type of renal cortex is the proximal tubule epithelial cell, from which renal cell carcinomas are thought to originate (9, 10).

[1] This work was supported by grants from the Norwegian Cancer Society (Landsforeningen mot kreft) and the Norwegian Research Council for Science and the Humanities.

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EXPERIMENTAL PROCEDURES

Materials. Cyclic [^{3}H]AMP (27 Ci/mmol) and [^{3}H]adenosine (23 Ci/mmol) were from the Radiochemical Centre, Amersham, Bucks, U. K., whereas [γ-^{32}P]ATP (about 2 Ci/mmol) was prepared by a modification of the method of Glynn and Chappell (11), and purified by DEAE-Sephadex (Pharmacia, Sweden) chromatography. The purity of [γ-^{32}P]ATP was checked by ascending chromatography on polyethyleneimine-thin-layer sheets (Polygram Cel 300, from Macherey Nagel & Co., Germany) developed in

0003-9861/78/1892-372$02.00/0

1.2 M LiCl, and by descending paper chromatography as described by Kleppe *et al.* (12). The radiochemical purity was 97% in both systems. The only radiochemical impurity detected cochromatographed with inorganic phosphate. Cyclic AMP, ATP, adenosine, 1-methyl-3-isobutylxanthine, bovine pancreatic ribonuclease (type IIA), hemoglobin (type I), and histone (type II) were from Sigma Chemical Co., St. Louis. Erythro-9-(2-hydroxy-3-nonyl)adenine was from Dr. H. Storck, Boehringer-Mannheim, Germany. DEAE-cellulose (DE-52), phosphocellulose (P-11), and filter paper discs (3 MM of 2.3-cm diameter) were from Whatman Biochemicals Ltd., Maidstone, Kent, U. K. and Ultrogel (ACA44) from LKB AB, Bromma, Sweden. Casein (according to Hammerstein) and trichloroacetic acid were from Merck Chemical Co., Darmstadt, Germany.

The biological material was obtained from patients undergoing nephrectomy (Table I). Immediately after the kidney had been removed, it was sectioned longitudinally and specimens from macroscopically visible-tumor tissue and samples from apparently normal renal cortex were plunged into liquid nitrogen. The time interval between ligation of the renal artery and freezing varied from 1 to 15 min. The tissue immediately surrounding the specimens removed for biochemical investigations was examined histologically and the tumor specimens were found, in all cases, to be surrounded by renal carcinoma tissue which was homogeneous except for the scattered occurrence of small, necrotic foci. The specimens of renal cortex were surrounded by renal cortical tissue without overt pathological alterations.

TABLE I

AGE AND SEX OF PATIENTS FROM WHOM MATERIAL WAS OBTAINED[a]

Patient	Age (years)	Sex
F.C.	55	♀
A.N.	81	♂
W.L.	70	♀
N.N.	45	♀
E.S.	64	♀
A.H.	58	♂
J.H.	65	♂

[a] All patients had normal renal function as judged from serum concentration of creatinine and other kidney function tests when performed. The size of the tumors varied from 2.5 × 2.5 × 2.5 cm to 4 × 4 × 4 cm, except for one large, locally invasive tumor (W.L.) compressing the remaining part of the kidney, so no sample of normal renal cortex was obtained. Another patient (N.N.), supposed to have a malignant tumor, appeared to have a non-neoplastic cyst, so only normal renal cortex was studied. The other patients had well defined tumors confined to one of the two poles of the kidney. One of the patients (J.H.) had cerebral and pulmonal metastases, the other patients had no recognized tumor metastasis at the time of operation. One of the tumors (F.C.) was classified as a "dark cell" carcinoma (13). The others were classified as "clear cell" carcinomas (13).

Preparation of tissue extract. The frozen tissue was thawed in homogenization buffer (10 mM Tris-HCl, pH 7.8, containing 1 mM EGTA,[3] 0.1 mM EDTA, 0.25 M sucrose, and 20 mM 2-mercaptoethanol), and homogenized (1/10, w/v) in an emulsifier (Silverson Mach. Ltd., Waterside, Bucks., U. K.) operated at full speed for 2 × 30 s, followed by two strokes at 465 rpm in a glass/Teflon (Thomas type C) homogenizer. The pH of the homogenate was adjusted to 7.6 by the addition of 1.5 M Tris-HCl, pH 8.8, and a high speed supernatant obtained by spinning at 20,000*g* for 20 min. Such supernatants, either obtained from carcinoma or cortex, contained 7 to 8 mg of protein/ml. In some preliminary experiments, the homogenization (1/4, w/v) was performed in 15 mM Tris-HCl, pH 7.65, containing 10 mM EDTA, 0.25 M sucrose, and 20 mM 2-mercaptoethanol. In one case, whole blood (with added EDTA) from one of the patients (A.H.) undergoing nephrectomy was centrifuged at 5000*g* for 10 min, the supernatant (plasma) saved, and the sediment (formed elements) resuspended in the homogenization buffer, homogenized, and a high speed supernatant obtained. All handling of tissue extracts was in the cold (0–4°C).

Preparation of isozymes of cyclic AMP-dependent protein kinase. Protein kinase I and protein kinase II were separated by DEAE-cellulose chromatography. The high speed supernatant (20 ml) was diluted with 1 vol of an aqueous solution of 1 mM EGTA, 0.1 mM EDTA, pH 7.6, and applied to a DEAE-cellulose column (20 × 1 cm) equilibrated with 15 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 20 mM 2-mercaptoethanol. The column was washed with 0.5 liter of the same buffer and the enzyme activities eluted with a linear gradient (0.3 liter of 0–350 mM NaCl) in the equilibration buffer. The peak fractions of protein kinase I and II were pooled separately, passed through phosphocellulose columns (1 × 2 cm) equilibrated with 15 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 20 mM 2-mercaptoethanol, and precipitated with 55% saturated ammonium sulfate. The pellets so obtained were dissolved in 15 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 20 mM 2-mercaptoethanol, and desalted by passage through Sephadex G-25 columns equilibrated with the same buffer. The preparations had binding capacities ranging from 35 to 75 pmol of cyclic AMP/mg of protein.

Measurement of cyclic [^{3}H]AMP binding. Bound and free nucleotide were separated by ammonium sulfate precipitation as described (14). In the presence

[3] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE II

EFFECT OF INCUBATION CONDITIONS ON THE AMOUNT OF CYCLIC [^{3}H]AMP BOUND AND STATE OF ACTIVATION OF PROTEIN KINASE ISOZYMES[a]

Isozyme form of protein kinase present	Concentration of cyclic [^{3}H]AMP	Agent added	15 min of incubation		120 min of incubation	
			Cyclic [^{3}H]-AMP bound (fmol/ml)	Activity ratio	Cyclic [^{3}H]-AMP bound (fmol/ml)	Activity ratio
I	1.5 nM		4	0.03	14	0.08
		800 mM NaCl	85	0.85	119	1.00
II	1.5 nM		31	0.25	66	0.31
		800 mM NaCl	26	0.27	74	0.30
		Histone (2 mg/ml)	45	0.48	94	0.61
		NaCl/histone	29	0.29	75	0.32
II	15 nM		50	0.47	114	0.58
		800 mM NaCl	49	0.28	156	0.43
		Histone (2 mg/ml)	183	0.64	413	0.94
		NaCl/histone	107	0.33	227	0.49

[a] Protein kinase I (diluted finally to a binding capacity for cyclic [^{3}H]AMP of 0.12 pmol/ml and a protein concentration of 3.4 μg/ml) and protein kinase II (binding capacity, 0.48 pmol/ml, protein concentration, 7.4 μg/ml) were incubated at 0°C in 15 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 20 mM 2-mercaptoethanol in a volume of 4 ml. The incubations contained either histone, histone and NaCl, serum albumin (0.5 mg/ml), or serum albumin and NaCl. The concentrations (final) of cyclic [^{3}H]AMP, histone, and NaCl are indicated in the table. Duplicate samples (0.6 ml) were removed for determination of cyclic [^{3}H]AMP bound and (0.1 ml) for determination of protein kinase activity ratio (ratio of kinase activity in the absence and presence of 2 μM cyclic AMP). The data shown were obtained with preparations of isozyme from renal carcinoma, but the results were similar for isozymes from renal cortex. In this particular experiment, the incubation time was 15 min and the blank values varied from 0.02–0.04% of the added (10^6 cpm) radioactivity, depending upon the concentration of salt and histone in the incubations. The percentage of the added radioactivity incorporated into substrate varied from 0.008% (protein kinase I assayed in the absence of cyclic AMP) to 1.0% (protein kinase II assayed in the presence of cyclic AMP).

of 800 mM NaCl and 1.5 nM cyclic [^{3}H]AMP, protein kinase I was near saturated with the nucleotide and completely dissociated after 2 h of incubation at 0°C, whereas protein kinase II showed only a modest degree of activation and saturation (Table II).

When cyclic [^{3}H]AMP binding preferentially to cyclic AMP-dependent protein kinase isozyme type I was to be assayed, the incubations contained 50 mM Hepes-NaOH, pH 7.2, 800 mM NaCl, 30 mM EDTA, 20 mM 2-mercaptoethanol, 0.1 mM 1-methyl-3-isobutylxanthine, 10 μM adenosine, 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine, 1.5 nM cyclic [^{3}H]AMP, and bovine serum albumin (0.5 mg/ml). Adenosine was present to prevent the binding of cyclic [^{3}H]AMP to binding proteins not related to cyclic AMP-dependent protein kinase (15–17), and 1-methyl-3-isobutylxanthine and erythro-9-(2-hydroxy-3-nonyl)adenine (18) was present to prevent the degradation of cyclic [^{3}H]AMP and adenosine, respectively.

In the presence of histone (2 mg/ml), 15 nM cyclic [^{3}H]AMP was sufficient to give more than 90% saturation (and activation) of both protein kinase II (Table II) and I (data not shown).

When binding of cyclic [^{3}H]AMP to both isozymes was to be measured, the incubations contained 15 mM Hepes-NaOH, pH 7.2, 1 mM EDTA, 20 mM 2-mercaptoethanol, 0.1 mM 1-methyl-3-isobutylxanthine, 10 μM adenosine, 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine, 30 nM cyclic [^{3}H]AMP, and histone (2 mg/ml).

The reactants were routinely allowed to incubate for 2 h at 0°C in a volume of 0.4 ml. The final concentration of binding sites during the incubations was kept below 0.5 pmol/ml, and linearity of amount of bound nucleotide *versus* sample concentration was then observed for all samples measured. For one experiment, the binding of cyclic [^{3}H]AMP was determined under the conditions for the assay of protein kinase activity.

Assay of protein kinase activity. This was routinely performed in 15 mM Hepes-NaOH, pH 7.0, containing 10 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 30 μM EDTA, 30 μM [γ-^{32}P]ATP (about 3 μCi/ml), histone (0.67 mg/ml), and (when added) 2 μM cyclic AMP. In some experiments, the concentration of ATP and histone was varied. Casein was substituted for histone as substrate, or various concentrations of salt were present. The incubations were conducted at 30°C in a volume of 0.15 ml, and terminated by pipetting samples (100 μl) on dry filter paper disks (19, 20) which had been presoaked in aqueous 10 mM Na_3PO_4, 10 mM $Na_4P_2O_7$, 1 mM ATP. After 1 min, the disks were dropped into ice cold aqueous 5% (w/v) trichloroacetic acid (10

ml/disk) containing 10 mM Na_3PO_4, 10 mM $Na_4P_2O_4$. The filter disks were washed for 15 h in the cold (2°C) with one change of the trichloroacetic acid-phosphate solution, and then for 2 h at room temperature with two changes of the same solution.

One unit of the kinase activity is defined as the activity incorporating 1 pmol of phosphate into acceptor protein (histone at 0.67 mg/ml or casein at 3 mg/ml) per min at 30°C. The protein kinase activity ratio is defined as the ratio of phosphate incorporated in the absence and presence of added (2 μM) cyclic AMP.

Very low kinase activities had to be measured in the experiment of Table II due to the presence of inhibitory concentrations of salt (21, 22) in some incubations and the low expression of kinase activity in the absence of added cyclic AMP in other incubations (see legend to Table II). For the accurate measurement of low activity of protein kinase, it was found desirable to explore the effects of incubation time, ionic strength, and type and concentration of substrate protein on the magnitude of the assay blanks. The blank values increased rapidly when the assay mixture (without added enzyme) was incubated at 30°C, probably because of electrostatic interaction between [γ-^{32}P]ATP and the basic protein histone, since the blank values were considerably lower at high ionic strength and when the acid protein casein was substituted for histone (Table III). To obtain low blank values, the [γ-^{32}P]ATP was added to the incubation mixture immediately before the reaction was started by the addition of enzyme, and the concentration of histone in the assays as well as the reaction time kept low. The samples were routinely assayed at two or more different concentrations to ensure linearity of kinase activity with respect to enzyme concentration. The samples were diluted so that the reaction rate (in the presence of 2 μM cyclic AMP) was linear with respect to time for at least 10 min. The reaction time was routinely 5 min.

TABLE III

EFFECT OF THE TYPE AND CONCENTRATION OF ACCEPTOR PROTEIN ON THE RADIOACTIVITY OF ENZYME BLANKS[a]

	Acceptor protein (mg/ml)	KCl (mM)	Blank value (% of total radioactivity)
None	—	—	0.0118 ± 0.00058
	—	150	0.0123 ± 0.00045
Casein	7	—	0.0305 ± 0.00053
	7	150	0.0179 ± 0.00028
Histone	0.7	—	0.0179 ± 0.00064
	0.7	150	0.0128 ± 0.00015
Histone	7	—	0.0533 ± 0.00072
	7	150	0.0251 ± 0.00081

[a] The [γ-^{32}P]ATP (30 μM) was incubated for 5 min in the presence of casein (7 mg/ml), histone (0.7 mg/ml or 7 mg/ml), or in the absence of substrate protein. All incubations were performed both in the absence and presence of 150 mM KCl. The results are given as mean ± S.D. (n = 4).

Endogenous phosphorylation of protein kinase II. The incubation conditions were essentially those described by Rangel-Aldao and Rosen (23) for autophosphorylation of protein kinase II. The preparations of protein kinase II were incubated in 15 mM Hepes-NaOH, pH 7.0, containing 0.1 mM EGTA, 30 μM EDTA, 5 mM $Mg(CH_3COO)_2$, 5 μM [γ-^{32}P]ATP, 20 mM 2-mercaptoethanol, and serum albumin (0.5 mg/ml) at 0°C in a volume of 0.32 ml. In some cases, 30 mM EDTA was present in the incubations. For determination of the amount of radioactive phosphate incorporated, samples (50 μl) were applied to Sephadex G-25 columns (0.4 × 6 cm) equilibrated with 15 mM Hepes-NaOH, pH 7.0, containing 100 mM EDTA, 100 mM NaF, 100 μM ATP, 20 mM 2-mercaptoethanol, and albumin (0.5 mg/ml). Protein kinase II was eluted quantitatively in a volume of 0.2 ml and precipitated with 10% (w/v) ice cold trichloroacetic acid. The resulting precipitate was collected on membrane filters and counted.

Sucrose density gradient centrifugation. Linear (5–20% w/v) sucrose gradients (10 ml) were overlayered with sample (0.15 ml) and spun for 18 h at 40,000 rpm in the SW 40 ti. rotor of a Beckman L2-65B ultracentrifuge. Fractions (0.55 ml) were collected. Bovine pancreatic ribonuclease and bovine hemoglobin were used as markers for S value determination. Hemoglobin was determined by its absorbance at 411 nm. Ribonuclease activity was determined by measuring the decrease in trichloroacetic acid-precipitable [^{3}H]RNA.

Protein. This was measured by the method of Klungsøyr (24) using bovine serum albumin as the standard.

RESULTS

Behavior of protein kinase isozymes of renal extracts on DEAE-cellulose chromatography: effect of extraction-conditions. The tissue specimens from the first two pairs of renal cortex/carcinoma investigated were homogenized (¼, w/v) in 15 mM Tris-HCl buffer containing 10 mM EDTA. DEAE-cellulose chromatography of such extracts produced a minor peak of cyclic AMP-dependent histone kinase activity with associated high affinity cyclic [^{3}H]AMP binding activity and a major peak associted with binding activity of lower affinity, corresponding to protein kinases I

and II, respectively (Fig. 1*a*). Cyclic AMP-independent kinase activity in the flow-through fractions and high affinity cyclic [^{3}H]AMP binding activity eluted between protein kinase I and II probably represented the free catalytic and regulatory moieties of protein kinase I since a preparation of that isozyme, after its dissociation by cyclic [^{3}H]AMP, gave rise to similarly chromatographing catalytic activity and binding activity. An experiment (Fig. 1*b*) was set up to see if prolonged dialysis against dilute phosphate buffer could afford the recombination of the subunits of protein kinase I in the tissue extract. The dialysis did not affect the total assayable kinase activity of the extract, but the sedimentation velocity (4.5 to 6S) of the kinase activity in sucrose gradients was lower than for freshly prepared extracts (5.5 to 7S), and the kinase activity was eluted from DEAE-cellulose at an ionic strength (Fig. 1*b*) intermediate between that required to elute protein kinase I and protein kinase II from freshly prepared extracts (Fig. 1*a*). Aging of tissue extracts resulted in a progressive reduction of protein kinase I with the concomitant appearance of a new peak of cyclic AMP-dependent protein kinase activity between protein kinase I and II. The new peak was associated with the high affinity cyclic [^{3}H]AMP binding characterizing protein kinase I. On prolonged aging of the extract, protein kinase II eluted at lower ionic strength, approaching the newly formed peak of kinase activity, resulting in an incomplete separation of the isozymes.

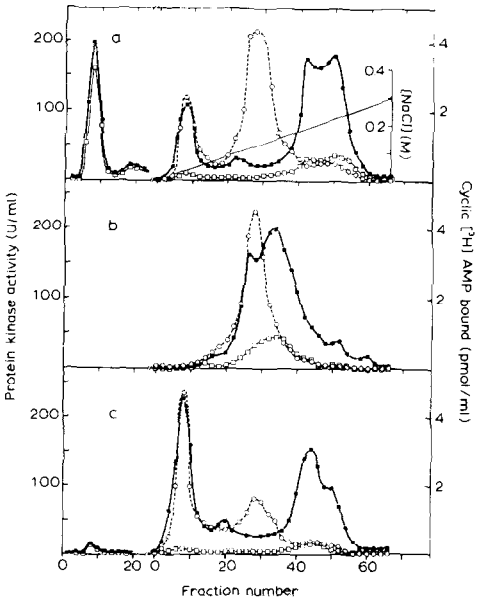

FIG. 1. DEAE-cellulose chromatography of variously prepared extracts of renal cell carcinoma. *a*, the carcinoma tissue (W.L.) was homogenized (1/4, w/v) in 15 mM Tris-HCl, pH 7.65, containing 10 mM EDTA, 20 mM 2-mercaptoethanol, and 0.25 M sucrose. The high speed supernatant of the extract was diluted with ice cold water to 5 times its original volume, and DEAE-cellulose chromatography performed as described under Experimental Procedures. *b*, a sample from the high speed supernatant (referred to above) was dialyzed for 24 h against 5 mM sodium phosphate buffer, pH 6.5, containing 0.1 mM EDTA and 20 mM 2-mercaptoethanol, with several changes of the buffer. The dialyzed solution was mixed with 4 vol of column equilibration buffer and chromatographed as above. *c*, a specimen from the same tumor was extracted (1/10, w/v) and chromatographed on DEAE-cellulose as described under Experimental Procedures. Fractions of 15 ml were collected. Protein kinase activity was assayed under standard conditions (see Experimental Procedures) in the absence (□——□) and presence (■——■) of (2 μM) cyclic AMP. The binding of cyclic [^{3}H]AMP (○- - -○) was assayed under conditions (high ionic strength, 1.5 nM cyclic [^{3}H]AMP favoring the selective binding of the nucleotide to the binding moiety of protein kinase I. The activities are given per ml of fraction.

In another experiment, the homogenization conditions were chosen to minimize dissociation of protein kinase I and proteolysis. Such conditions did favor the holoenzyme form of protein kinase I (Fig. 1*c*) and were, therefore, used for the rest of the study on isozyme distribution. The amount of histone kinase activity in the flow through fractions (to be ascribed to the free catalytic moiety of the protein kinases) was always less than 3% of the total activity, and 85 to 95% of the total activity eluted as expected for protein kinases I or II, while the remaining activity (probably representing partially proteolyzed protein kinase I) was eluted between the two major isozyme peaks.

Comparison of the protein kinase isozyme distribution in renal cortex and carcinoma. Both protein kinase I and protein kinase II were present in the extracts of renal cortex (Fig. 2) and carcinoma (Fig. 3)

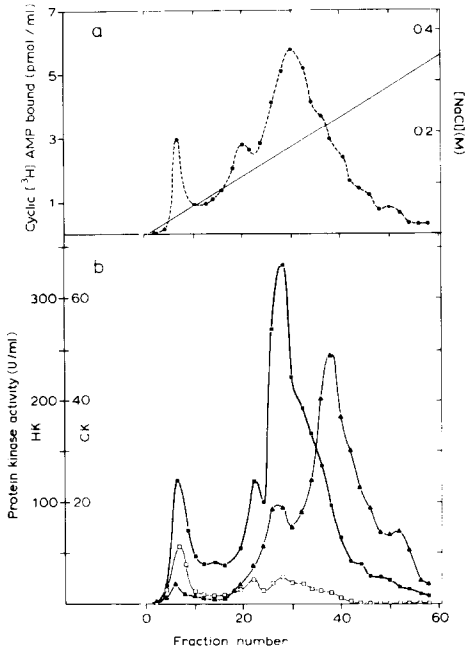

FIG. 2. DEAE-cellulose chromatography of renal cortex extract. Apparently normal renal cortex from the lower pole of a kidney (J. H.) with a renal cell carcinoma at its upper pole, was extracted and chromatographed as described under Experimental Procedures. One hundred-forty one milligrams of protein were loaded on the column. Fractions of 5 ml were collected. *a*, cyclic [^{3}H]AMP binding activity (●- - -●) was measured under conditions (histone, 30 nM cyclic [^{3}H]AMP) when both isozyme forms of cyclic AMP-dependent protein kinase were saturated with the nucleotide. *b*, the phosphotransferase activity of desalted fractions was measured with histone as the substrate in the absence (□——□) and presence (■——■) of (2 μM) cyclic AMP or with casein (6 mg/ml) as the substrate in the presence of 150 mM KCl (▲——▲).

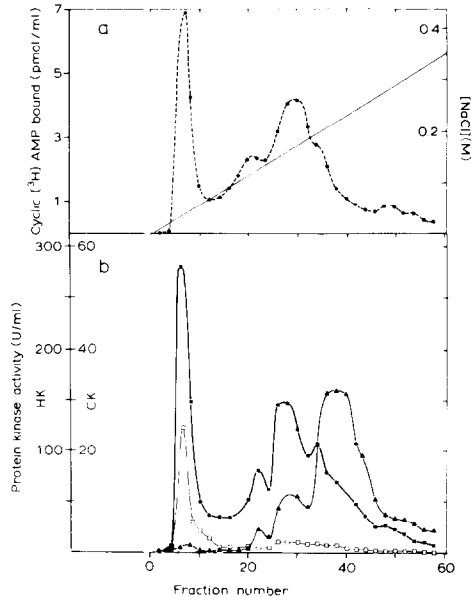

FIG. 3. DEAE-cellulose chromatography of renal carcinoma extract (139 mg of protein). A specimen from the renal cell carcinoma of the kidney (J.H.) referred to in the legend to Fig. 2 was prepared for chromatography and measurement of binding and kinase activities as described in the legend to Fig. 2.

analyzed by DEAE-cellulose chromatography. The ratio of protein kinase I to protein kinase II varied from 0.18 to 0.28 in renal cortex and from 0.36 to 0.91 in renal carcinoma. In the three pairs of renal cortex/carcinoma studied, the proportion of protein kinase I to protein kinase II was about two times higher in the extracts of carcinomas than in the renal cortex extracts (Table IV).

For the two first pairs (F.C. and A.N.) of renal cortex/carcinoma (homogenized under conditions resulting in dissociation of protein kinase I, and not included in Table

TABLE IV

PROTEIN KINASE ISOZYME DISTRIBUTION IN RENAL CORTEX AND CARCINOMA[a]

	I/II
Renal Cortex (E.S.)	0.18 (0.28)
Renal carcinoma (E.S.)	0.36 (0.53)
Renal cortex (A.H.)	0.17 (0.28)
Renal carcinoma (A.H.)	0.38 (0.54)
Renal cortex (J.H.)	0.25 (0.36)
Renal carcinoma (J.H.)	0.54 (0.80)
Renal cortex (N.N.)	0.28 (0.33)
Renal carcinoma (W.L.)	0.90 (1.11)
Renal carcinoma (W.L.)	0.92 (1.22)

[a] The conditions for preparation of tissue extracts and DEAE-cellulose chromatography were as described under Experimental Procedures. The histone kinase activity (in the presence of 2 μM cyclic AMP) of the DEAE-cellulose fractions was plotted on graph paper, the areas corresponding to protein kinase I, protein kinase II, and the kinase activity eluted between the two major isozymes cut out and weighed. The ratio between the activity ascribed to protein kinase I and II is given for each tissue extract. One of the renal carcinomas (W.L.) was chromatographed at two different occasions. The ratio between protein kianse I + kinase activity eluted at intermediate ionic strength and protein kinase II is given in parentheses.

IV), the free cyclic AMP binding activity was higher for the carcinoma extracts (Fig. 3*a*) than for the cortex extracts, suggesting that protein kinase I was relatively more abundant also in those carcinomas. Furthermore, a specimen from a large locally invasive tumor contained relatively more protein kinase I than a specimen of normal cortex from a noncarcinomatous kidney (Table IV). Since the kinase activity eluted between the two main protein kinase isozymes probably represented partially proteolysed protein kinase I (see preceeding section), the ratio between the sum of those two kinase activities and the activity due to protein kinase II was calculated (numbers in parentheses in Table IV). The ratios so obtained were also higher for the carcinomas than normal renal cortex. There was thus no indication that protein kinase I was more extensively proteolyzed in renal cortex than in the carcinomas. No detectable kinase activity was eluted by DEAE-cellulose chromatography of human serum, and very low activity, mainly protein kinase II, by chromatography of an extract of the formed elements of blood. The possibility that the protein kinase I activity in renal extracts was due to stagnant blood in the tissue could thus be ruled out. The recovery of cyclic AMP-dependent protein kinase activity from the DEAE-cellulose columns was 35–50% of the kinase activity recovered on sucrose density gradient centrifugation, raising the possibility that selective instability of protein kinase II from tumor on DEAE-cellulose chromatography might explain the high ratio of protein kinase I relative to protein kinase II in that tissue.

Tissue extract from two of the pairs of renal cortex/carcinoma (A.H., J.H.) were analyzed by sucrose density gradient centrifugation. The cyclic AMP-dependent protein kinase activity and the cyclic AMP binding activity co-sedimented at 6–7S. Comparison of the ratio of cyclic [^{3}H]AMP binding activity in the sucrose gradient fractions under conditions favoring binding to protein kinase I (high ionic strength, low nucleotide concentration) and conditions saturating both isozymes with the nucleotide (see Table II), indicated that the tumor extracts had a higher concentration of protein kinase I than had the renal extracts (data not shown), suggesting that the observed isozyme patterns were not due to artifacts during DEAE-cellulose chromatography.

A minor peak of kinase activity preferentially phosphorylating casein eluted like protein kinase I, whereas two major peaks eluted from DEAE-cellulose at slightly higher ionic strength than did protein kinase II (Figs. 2 and 3). The two main peaks were stimulated (about 100%) by the inclusion of 150 mM KCl in the incubations, but not by 2 μM cyclic AMP. The bulk of the casein kinase activity of both renal extracts and tumor extracts sedimented at about 10S, with a minor peak at 4S. The cyclic AMP dependence of the histone kinase activity in the DEAE-cellulose fractions containing protein kinase I was often less than the cyclic AMP dependence of protein kinase II (Figs. 2 and 3), but the preparations of protein kinase I after phosphocellulose chromatography were highly dependent on cyclic AMP for histone kinase activity (Table II).

Comparison of the amount of soluble cyclic AMP-dependent protein kinase activity in the extracts of cortex and carcinoma was done by measurement of the activity sedimenting at 4–8S in sucrose gradients loaded with renal cortex or carcinoma extracts. The histone kinase activity (measured in the presence of 2 μM cyclic AMP) per mg of tissue extract protein applied, differed by less than 5% for the two cases (A.M. and J.H.) investigated.

Linearity of protein kinase activity *versus* concentration of sample measured, ensured that the heat-stable protein kinase inhibitor did not influence the assay (25).

Cyclic AMP-dependence of the protein kinase isozymes: correlation with binding of cyclic AMP. When tested at low enzyme concentrations (about 50 units/ml), the preparations of protein kinase I were more cyclic AMP-dependent than those of protein kinase II. At higher enzyme concentrations (about 300 units/ml), both preparations were activated at least 20-fold by 2 μM cyclic AMP under standard phosphotransferase assay conditions.

In the experiment of Fig. 4, protein kinase

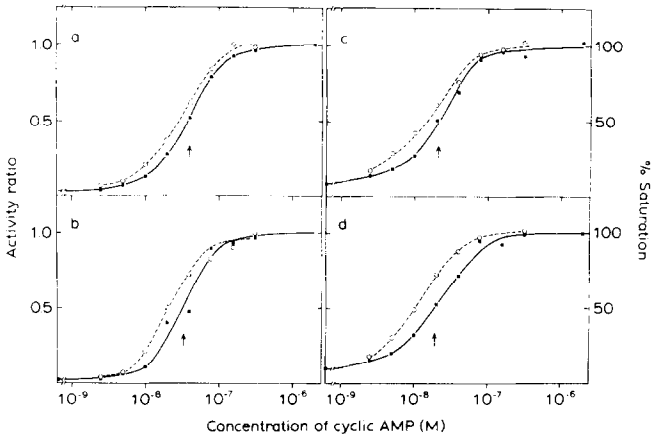

FIG. 4. Activity ratio and amount of cyclic [^{3}H]AMP bound to protein kinase I and II from renal cortex and renal cell carcinoma. Cyclic AMP-dependent protein kinases type I and II were prepared from normal renal cortex and renal cell carcinoma as described under Experimental Procedures, and incubated under standard phosphotransferase assay conditions in the presence of the concentrations (total) of cyclic [^{3}H]AMP indicated on the abscissa. After 10 min of incubation, samples (200 μl) were removed for the determination (in duplicate) of the amount of cyclic [^{3}H]-AMP bound (O- - -O) and other samples (100 μl) for the duplicate measurement of protein kinase activity in the absence and presence of added (2 μM) unlabeled cyclic AMP for calculation of activity ratio (■——■). The presence of tritiated nucleotide in the assay mixture did not affect the measurement of phosphate-incorporation into histone. The contribution of ^{32}P trapped on the Millipore-filters to the counts per min in the ^{3}H channel of the scintillation counter was corrected for to determine the amount of cyclic [^{3}H]AMP bound. *a*, protein kinase I from renal cortex (final binding capacity for cyclic AMP, 1.2 pmol/ml); *b*, protein kinase I from renal cell carcinoma (1.7 pmol/ml); *c*, protein kinase II from renal cortex (1.8 pmol/ml); *d*, protein kinase II from renal cell carcinoma (1.4 pmol/ml).

was incubated in the presence of various concentrations of cyclic [^{3}H]AMP under protein kinase assay conditions and aliquots removed 5 min (not shown), 10 min (Fig. 4), and 15 min (not shown) after the start of the incubations, for the determination of the amount of cyclic [^{3}H]AMP bound and the degree of activation of the kinase. No difference in the concentration of cyclic [^{3}H]AMP needed to half-maximally activate the kinase activities or half-maximally saturate the binding sites was noted at any time point between kinase derived from normal renal cortex or renal cell carcinoma. Protein kinase I from both sources required higher concentrations of cyclic AMP than protein kinase II to reach a certain degree of activation or saturation (Fig. 4).

Both the activity ratio and the amount of cyclic AMP bound increased from 5 to 15 min of incubation at concentrations of the nucleotide giving intermediate degrees of kinase activation. Whereas the measurement of bound cyclic [^{3}H]AMP represents the amount bound at the end of the incubation period, the phosphotransferase activity measured represents the accumulated phosphate incorporation into substrate during the whole incubation period, the degree of activity of the kinase being lower in the first part of the period. The slight discrepancy between the degree of saturation of cyclic [^{3}H]AMP binding sites and the activity ratio at subsaturating concentrations of the nucleotide (Fig. 4) thus does not indicate a dissociation of the ability of cyclic AMP to bind to and to activate protein kinases.

Endogenous phosphorylation of protein kinase II. Endogenous phosphorylation was found to occur in preparations of protein kinase II from all the pairs of renal cortex/carcinoma investigated (J.H., E.S., A.H.), whether the preparation was from the first, middle, or last part of the peak of

protein kinase II eluted from DEAE-cellulose. A typical time pattern of the reaction is shown in Fig. 5, which also demonstrates the Mg^{2+} dependence of the reaction (complete inhibition of phosphate incorporation when EDTA was added). Sucrose gradient centrifugation of the endogenously phosphorylated mixture showed co-sedimentation of protein-bound ^{32}P and cyclic AMP-dependent protein kinase activity. The concomitant shift of cyclic [^{3}H]AMP binding activity and protein-bound ^{32}P on a gradient containing cyclic AMP (2 μM) strongly suggested that the regulatory moiety of protein kinase was the main substrate for the endogenous phosphorylation. Similar shifts were observed when the endogenously phosphorylated preparations were chromatographed on Ultrogel in the absence and presence of cyclic AMP.

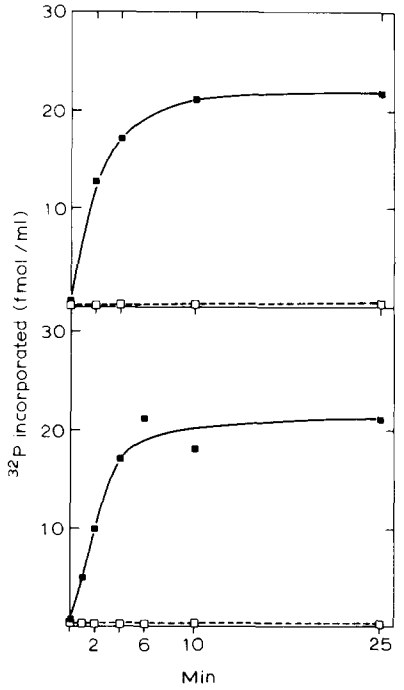

FIG. 5. The endogenous phosphorylation in preparations of protein kinase II from renal cortex and carcinoma. Protein kinase II was incubated at 0°C in the presence of 5 μM [γ-^{32}P]ATP and Mg^{2+} with (□- - -□) or without (■——■) EDTA, and the amount of trichloroacetic acid-precipitable ^{32}P determined (see Experimental Procedures) after the periods of time indicated on the abscissa. The upper part of the figure shows the time course of endogenous phosphorylation of a preparation of protein kinase II from renal cortex. The lower part of the figure shows the phosphorylation of protein kinase II from renal carcinoma.

DISCUSSION

Any consistent alteration of the protein kinase isozyme pattern under certain physiological or pathological conditions may give clues to the biological significance of the presence of two discrete (2, 26, 27) isozymes.

Altered distribution of the two main isozyme forms of cyclic AMP-dependent protein kinase has been noted during development (28), in various phases of the cell cycle (29), in response to estradiol (30), and in cardiac hypertrophy (31). The complete absence of protein kinase I and its associated high affinity binding protein in hepatoma cell lines (5, 32, 33) contrasts with the presence of that isozyme in hepatocytes (34) and rat liver (5, 35, 36), and raised the question whether human tumors may show similar isozyme aberrations. The present study revealed a higher concentration of protein kinase I in all the human renal cell carcinomas investigated than in nontumorous renal cortex (Table IV). Protein kinase I was only apparent (Fig. 1*c*) when homogenization conditions were such as to prevent dissociation of the holoenzyme into subunits (Fig. 1*a*) and retard proteolysis (Fig. 1*b*). An extract of the formed elements of blood contained mostly protein kinase II, reducing the likelihood that protein kinase I of tumor extracts stems from trapped blood or from cells derived from the blood (granulocytes, lymphocytes, macrophages) and known to be present in appreciable quantities in solid tumors (37, 38).

The properties of the protein kinase isozymes from normal and carcinoma tissue were remarkably similar, regarding cyclic AMP dependence of the kinase activity and the concentration of cyclic AMP needed to give half-maximal activation and saturation of the enzymes (Fig. 4). There was thus no indication of abnormal interaction between cyclic AMP and protein kinases from renal cell carcinoma *in vitro*. Recent studies (T. M. Fossberg and S. O. Døskeland, unpublished observations) have demonstrated an elevated intracellular degree of activation

of the cyclic AMP-dependent protein kinase activity from carcinomas with elevated cyclic AMP levels, indicating that cyclic AMP activates the protein kinases of tumor cells also *in vivo*. The time course of the endogenous phosphorylation of protein kinase II was the same whether the enzyme preparation had been obtained from renal cortex or renal cell carcinoma, and was similar to that reported for protein kinase II from bovine beart (23) indicating that protein kinase II of tumor origin is subject to autophosphorylation like protein kinase II from several nonmalignant tissues (39, 40). The present study thus failed to demonstrate any impairment of the enzymes carrying out cyclic AMP-dependent phosphorylation in renal carcinomas.

ACKNOWLEDGMENTS

We are indebted to Drs. T. Gjersvik, P. Å. Høisæter and M. Farstad for their collaboration in obtaining the specimens of renal tissue, to Dr. K. A. Abraham for help to synthesize [γ-^{32}P]ATP and to Dr. A. J. Lillehaug for checking the purity of [γ-^{32}P]ATP by paper chromatography. The *in vivo* tritium-labeled E. Coli RNA was a gift from Dr. L. Haarr.

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