PROTEIN KINASES IN HEPATOMA, AND ADULT AND FETAL LIVER OF THE RAT*

I. SUBCELLULAR DISTRIBUTION

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Received September 8,1975

SUMMARY

Comparison of the subcellular distribution of protein kinases in hepatoma, adult and fetal liver showed that nuclei of growing tissues contain a 3 to 5 times higher percentage of the activity in whole homogenate than nuclei of adult liver. The cytoplasmic protein kinase in hepatoma and fetal liver was stimulated by cAMP** only half as much as that in adult liver. The nuclear activity was unresponsive to cAMP in all three tissues. Subfractionation of nuclei gave a final preparation that was more active with endogenous substrate than with added histone as phosphate acceptor. The hepatoma nuclei contained latent activity that could be unmasked in the presence of Triton X-100, those of adult and fetal liver did not. A partial resolution of the nuclear activity on DEAE-cellulose is reported.

INTRODUCTION

The turnover of the phosphoryl moiety of phosphoproteins has been recognized for some time as an important regulatory mechanism of cellular functions (1). There is now ample evidence that many hormone-induced and probably all cAMP-mediated changes of metabolic activities in tissues and cells involve the phosphorylation of enzymes and other key proteins (2-6). The onset of DNA synthesis and subsequent cellular proliferation after administration of an appropriate stimulus to quiescent cells in culture (7-11), as well as the regeneration of the liver after partial hepatectomy (12), appear to be preceded by phosphorylation of nuclear proteins. Thus, phosphorylation of key proteins seems to play a major role in the regulation of many metabolic functions and of cell growth; both these controls appear to be deranged or lost in tumors (13).

A comparative study of the enzymes that carry out the phosphorylation of proteins, the protein kinases, in a rapidly growing hepatoma and in adult and fetal liver seemed therefore of interest, and forms the subject of this report.

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* This work was supported by USPHS Grant No. 7Rol-CA-16033 from The National Institutes of Health.

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**Abbreviations used: cAMP, adenosine 3',5'-monophosphate.

MATERIALS AND METHODS

The Morris hepatoma 7288C used in this study was obtained through the courtesy of Dr. Sidney Weinhouse and was propagated bi-weekly in 8-week old male rats of the Buffalo strain. Adult and fetal animals were of the same strain; fetuses were between 17 and 18 days of gestation.

Gamma-^{32}P-ATP with a specific activity of about 20 Ci/mmol was obtained from New England Nuclear. ATP and dithiothreitol were obtained from Calbiochem, cAMP and histone fraction II-A from Sigma Chemical Company, theophylline was purchased from Nutritional Biochemicals Corporation.

Preparation of subcellular fractions: Eight-week old male rats were killed by decapitation and the livers were perfused in situ with 0.32 M sucrose, containing 20 mM Tris-HCl pH 7.4 and 3 mM $MgCl_2$ (homogenizing medium) to prevent contamination of the nuclei with erythrocytes. Fetal livers were perfused after excision. All subsequent operations were carried out at 0° to 4° C. Livers were homogenized in three volumes of ice-cold homogenizing medium and nuclei were purified according to Widnell and Tata (14). The postnuclear supernate refers to that obtained after centrifugation at 700 x g. Tumors were excised 2 1/2 weeks after implantation, carefully freed of necrotic material and homogenized as described above. The nuclei from hepatoma were prepared essentially in the same fashion as those from livers, except that the final pelleting was done in 2.2 M sucrose because the yield of nuclei from hepatoma was poor when that step was done in 2.4 M sucrose. The nuclei were washed once in 0.1 M KCl containing 2 mM EDTA to remove soluble enzymes and were then extracted with 0.2 % Triton X-100. The subfractionation of the nuclei is described in detail in the legend to table III.

Enzyme assay and definition of unit: The enzymes were assayed essentially according to Yamamoto et al. (15), except that 50 nanomoles ATP containing from 1 to 2 x 10^6 cpm were used in the incubation mixture. Incubation was for 10 min at 30° C in a total volume of 0.2 ml; the reaction was stopped by the addition of 0.1 ml ice-cold 12.5 mM ATP, followed by 4 ml of 25 % TCA; 0.15 ml of BSA (10 mg/ml) were then added as carrier protein, and the tubes were left standing on ice for 30 min to ensure complete precipitation before filtering through Whatman GF/A glass fiber filters. The filters were washed twice with 6 ml 10% TCA, dried and counted in a Beckman LS-233 liquid scintillation counter. One unit is defined as the amount of enzyme that transfers one nanomole of phosphate from ATP to an acceptor protein in one minute under the conditions of the assay. Protein determinations were done by the method of Lowry (16); DNA was measured by the method of Burton (17).

Column Chromatography DEAE-cellulose (DE-52, Whatman) was suspended in 1.0 M Tris-HCl, pH 7.2, and the finer particles were poured off several times, each time diluting the buffer with distilled water. A column of 3 ml packed volume (0.6 x 12.5 cm) was then poured and equilibrated with 20 mM Tris-HCl, pH 7.2, containing 0.5 mM dithiothreitol.

RESULTS AND DISCUSSION

A comparison of protein kinase activity in the rapidly growing Morris hepatoma 7288 C and in adult and fetal liver of the rat (Table I) shows that, although total activity per g fresh tissue does not differ greatly among these tissues, the subcellular distribution does vary significantly. Whereas the nuclei derived from adult liver contain only between 3 to 4% of the activity found in the whole homogenate, those from hepatoma contain between 13 to 15% and nuclei from fetal liver contain between 10 to 12%. Although total activity per g tissue varied considerably from experiment to experiment in the tumor as

Table I

Subcellular distribution of protein kinase activity in the Morris Hepatoma 7288 C, adult and fetal liver of the rat*

Cell fraction	Enzyme activity mμmol/g/min	% Distribution of enzyme activity	Protein mg/g fresh tissue	Specific activity mμmol/mg protein	DNA mg/g fresh tissue	% Nuclei Recovered
Liver:						
homogenate	21.6 ± 1.5 (6)	100	240 ± 17	0.09	2.26 ± 0.14	100
postnuclear supernate	16.8 ± 1.1 (6)	78	167 ± 13	0.10	0	0
purified nuclei	0.8** ± 0.05 (6)	3.7	9.3 ± 0.4	0.086	1.65 ± 0.20	73
Hepatoma:						
homogenate	31.0 ± 5.7 (4)	100	218 ± 21	0.142	5.1 ± 0.62	100
postnuclear supernate	22.0 ± 2.9 (4)	71	147 ± 15	0.15	0	0
purified nuclei	4.25** ± 0.63 (4)	13.7	20.4 ± 1.6	0.21	4.4 ± 0.51	86
Fetal liver:						
homogenate	18.7 ± 1.9 (3)	100	197 ± 21	0.095	3.43 ± 0.29	100
postnuclear supernate	12.3 ± 1.3 (3)	66	134 ± 11	0.092	0	0
purified nuclei	2.0** ± 0.4 (3)	10.7	10.8 ± 0.9	0.185	2.44 ± 0.21	71

* Cell fractions were prepared as described under "Methods."

** Corrected for yield of nuclei in terms of DNA recovered.

Values represent the mean ± S.D. of the number of experiments indicated in parenthesis.

reflected in the relatively large standard deviation, the subcellular distribution was remarkably reproducible. The greater concentration of enzyme activity in the nuclei derived from hepatoma and fetal liver is reflected in the 2 to 3 times higher specific activities in these fractions; both enzyme activity and protein content were corrected for the yield of purified nuclei in terms of DNA recovered in that fraction from whole homogenates. These results are conconant with those of many investigators (6-11) who have observed an increased phosphorylation of nuclear proteins in actively growing tissues and cells, compared to that in non-dividing tissues.

The response of protein kinase activity in the subcellular fractions derived from hepatoma, adult and fetal liver to cAMP at a final (optimal) concentration of 5×10^{-7} M in the incubation mixture is shown in Table II. The stimulation of enzyme activity in the homogenates and postnuclear supernates derived from hepatoma and fetal liver is only about half of that in the same fractions obtained from adult liver. This result agrees qualitatively, but not quantitatively, with the finding of Granner (18) that the stimulation of protein kinase in homogenates of HTC cells grown in culture is only about 1/5 that of adult liver. The greater difference between the response to the cyclic nucleotide of HTC cells (which are derived from the same Morris hepatoma 7288 C

Table II

Response of protein kinase activity in subcellular fractions to cyclic AMP*

Cell Fraction	Activity, nanomoles/g/min		Percent Stimulation
	-cAMP	+cAMP	
Liver:			
homogenate	21.6 ± 1.5 (6)	67.0, 81.0	310, 370
postnuclear supernate	16.8 ± 1.1 (6)	62.0, 71.0	370, 420
purified nuclei	0.8 ± 0.05(6)	1.04, 1.12	130, 140
Hepatoma:			
homogenate	31.0 ± 3.0 (4)	49.5, 65.0	160, 210
postnuclear supernate	22.0 ± 1.9 (4)	42.0, 53.0	190, 240
purified nuclei	4.25 ± 0.33(4)	5.1, 5.5	120, 130
Fetal Liver:			
homogenate	18.7 ± 1.6 (3)	35.6	190
postnuclear supernate	12.3 ± 0.9 (3)	28.2	230
purified nuclei	2.0 ± 0.2 (3)	2.6	130

* The concentration of cAMP used was 5×10^{-7} M.

used in this study) and normal liver may perhaps reflect a characteristic of cell growth in vitro as well as a characteristic of malignancy. It is also apparent from Table II that purified nuclei from all three tissues are least responsive to cAMP of all subcellular fractions examined. This may be due to the heterogeneity of nuclear protein kinases reported by Ruddon and Anderson (19). The response to cAMP in resuspended whole nuclei may thus represent an average response of several distinct enzyme species, some of which may be stimulated while others may be inhibited, and does not, therefore, permit a conclusive interpretation.

This possibility was investigated by subfractionating the purified nuclei, using as criteria for the existence of distinct enzyme species a) substrate preference for either added histone or endogenous phosphate acceptors; b) separability of distinct enzyme species of nuclear activity extracted with buffer containing 0.2% Triton X-100 on DEAE-cellulose; c) differential heat-sensitivity

Table III

Protein phosphokinase activity and substrate preference in nuclear fractions

Nuclear Fractions	Activity nanomol/g/min		
	+ Histone (A)	- Histone (B)	B/A
Liver			
1. Nuclei suspended in KCl-EDTA	0.80	0.50	0.63
2. KCl-EDTA extract	0.16	0.0	0
3. Salt-extracted pellet resuspended in Triton X-100	0.66	0.52	0.78
4. Triton X-100 extract	0.40	0.18	0.45
5. Triton pellet, resuspended	0.26	0.28	1.1
Hepatoma			
1. Nuclei suspended in KCl-EDTA	3.4	2.07	0.61
2. KCl-EDTA extract	0.89	0.22	0.25
3. Salt-extracted pellet resuspended in Triton X-100	5.55	4.20	0.76
4. Triton X-100 extract	1.60	0.52	0.31
5. Triton pellet, resuspended	4.42	5.8	1.3
Fetal Liver			
1. Nuclei suspended in KCl-EDTA	1.8	1.1	0.62
2. KCl-EDTA extract	0.59	0.17	0.29
3. Salt-extracted pellet resuspended in Triton X-100	1.2	0.9	0.75
4. Triton X-100 extract	0.7	0.19	0.27
5. Triton pellet	1.0	1.2	1.2

The nuclei, purified as described in Methods, were resuspended with homogenization in 0.1 M KCl containing 2 mM EDTA (2 ml per g tissue from which these were derived) (fraction 1); they were then centrifuged at 100,000 x g for 60 min in a Spinco Model L-2; the supernate is fraction 2; the pellet was resuspended with homogenization in the same volume of 20 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100, and let stand on ice, with occasional vortexing, for three hours (fraction 3). The suspension was then centrifuged, as described above, and the supernate was dialyzed overnight with at least two changes of the dialysate, against 20 mM Tris-HCl, pH 7.4, containing 0.5 mM dithiothreitol, and is referred to as Triton extract (fraction 4). The pellet was resuspended in the original volume of 20 mM Tris-HCl, pH 7.4, with homogenization, and is referred to as the Triton pellet (fraction 5).

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of DEAE-cellulose fractions; d) response to cAMP of DEAE-cellulose fractions.

The substrate preference of subnuclear fractions, expressed as the ratio of activity in the absence of added histone (B) to that in the presence of

added histone (A) is shown in Table III. The B/A ratio is remarkably similar for corresponding fractions in all three tissues studied. Both the KCl-EDTA and the Triton extracts have less than 45% activity without added histone. Whole nuclei have about 60% of maximal activity in the absence of added histone, the KCl-EDTA extracted pellets express 75 to 78% of maximal activity without added histone and the Triton extracted pellets are actually slightly inhibited by added histone. This progression toward independence of exogenous phosphate acceptor upon repeated extraction may reflect a sequential removal of histone kinases as well as increasing availability of tightly chromatin-bound non-histone phosphate acceptor proteins due to a loosening of the chromatin structure in the presence of the detergent. The data summarized in Table III are representative of three such experiments which all gave identical results. It is noteworthy that only in nuclei derived from hepatoma did the salt-extracted pellet resuspended in Triton and the pellet after Triton extraction give significantly higher activities than whole nuclei suspended in buffer alone, indicating substantial amounts of masked, possibly membrane-associated, activity in the nuclei of the tumor. Hildebrand and Tobey (20) have recently reported the isolation and characterization from actively dividing cells of DNA-lipoprotein complexes thought to be involved in the initiation of DNA synthesis; these complexes can be dissociated by detergents to release the DNA with a concomitant solubilization of the lipoprotein. The possibility that such complexes can be isolated from hepatoma and may contain protein kinase activity is presently being investigated in our laboratory.

The resolution of protein kinase activities extracted from the nuclei of hepatoma and adult liver with buffer containing 0.2% Triton X-100 on DEAE-cellulose is shown in Fig. 1. Fetal liver nuclei did not yield sufficient material to permit a similar analysis. The extract from hepatoma was resolved into five distinct peaks, whereas only three peaks were obtained from liver. The fractions comprising each peak were pooled and assayed for their stability to incubation at 45°. The corresponding fractions from liver and hepatoma had very similar heat inactivation curves: fraction I was totally inactivated in 20 min, with a half-life of 3 min; fraction II (absent from liver) had a half-life of 6 min; and fraction III gave a tri-phasic heat-inactivation curve, in that the activity seemed stable for 5 min at 45° but declined thereafter with a half-life of 8 to 9 min to 40% of the initial activity; this remaining activity was not inactivated even after 60 min at 45°, indicating that peak III is probably heterogeneous. None of the fractions responded to cAMP in the range of 5×10^{-5} to 5×10^{-7} M, perhaps as a result of the Triton treatment.

The results presented provide evidence that a) actively growing tissues contain a higher percentage of their total protein kinase activity in the

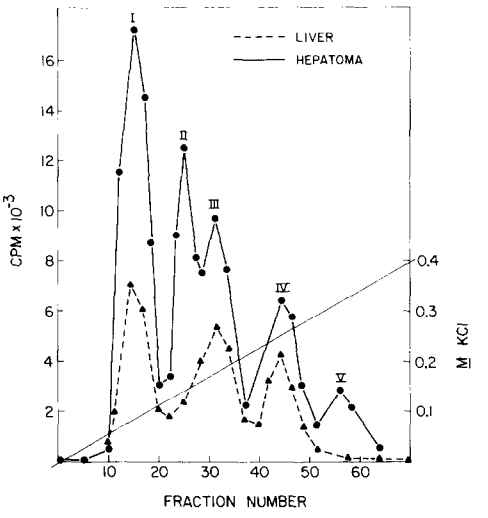

Fig. 1. The resolution of nuclear protein kinase activity on DEAE-cellulose. Three mg protein of the Triton extract of each, liver and hepatoma, were loaded onto two identical columns prepared as described in Methods and developed simultaneously. A single gradient maker provided the developing solution for both columns. Fractions of 0.4 ml were collected.

nucleus than the parent non-growing organ; b) the cytoplasmic protein kinase in hepatoma and fetal liver is less sensitive to regulation by cAMP than it is in adult liver. These observations are consonant with previous findings (21) that the isozyme distribution of several enzymes is similar in tumors and fetal tissues and differs from that of the cognate adult organ, and that the isozymes that are lost from tumors and have not yet deveoped in the embryonic organ are those that are under hormonal (or metabolite) control in the adult organ (22). While the cytoplasmic and nuclear protein kinases may not be isozymes in the strict sense, because they most likely have different physiological phosphate acceptor substrates, the characteristics found in hepatoma and fetal liver which differ from those in adult liver may nevertheless reflect the same phenomenon, namely, that the enzymic complement of tumors and embryonic tissues is geared above all toward rapid proliferation, perhaps at the expense of stringent physiological regulation.

ACKNOWLEDGEMENTS

I wish to thank Dr. Sidney Weinhouse, Fels Research Institute, Temple University, Philadelphia, Pa. for his generous gift of rats bearing the Morris hepatoma 7288C. The able technical assistance of Ms. Caryle Hirschberg and Ms. Janice A. Kolberg is gratefully acknowledged.

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