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PREREPLICATIVE MODULATION OF NUCLEAR PROTEIN KINASES IN THE REGENERATING RAT LIVER

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SUMMARY Partial hepatectomy in the rat is followed by a significant increase of nuclear protein content and by an increase of total nuclear protein kinase activity. Total nuclear protein kinase activity is maximal 16 hours after partial hepatectomy. Analysis of nuclear 0.35 M NaCl extracts by DEAE-Sephacel chromatography reveals the presence of four species of protein kinase, two of which are of the cAMP-dependent type. By 16 hours after partial hepatectomy the two nuclear cAMP-dependent protein kinase activities have increased several fold over the kinase activities found in liver nuclei of sham-operated controls. These data indicate a prereplicative modulation of nuclear protein kinase activities following the second wave of cAMP accumulation in the regenerating rat liver.

The process of rat liver regeneration occurring as the result of partial hepatectomy (PH) causes the liver remnant to shift from a state of minimal growth to one of rapid hypertrophy and hyperplasia. In the regenerating liver cAMP has been implicated as a positive regulator of proliferation on the basis of several findings, including: (a) a biphasic rise of cAMP levels (1) and activation of cytoplasmic cAMP-dependent protein kinase (2) precede the initiation of DNA synthesis, and (b) experimental conditions which bring about a delay of the second wave of cAMP accumulation, normally occurring between 12 and 14 hours after surgery, also cause a delay of DNA synthesis of equal duration (3).

The initiation of DNA synthesis in the previously quiescent hepatic tissue implies a derepression by cAMP of genetic information dormant in the resting state. The molecular mechanism of this derepression is unknown, but several lines of indirect evidence suggest that nuclear nonhistone proteins play a major

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role in controlling genetic information (4) and that phosphorylative modification of these proteins by nuclear protein kinases may modulate their activity as effectors of gene expression (5-9). Since cAMP exerts its biological effects through the activation of cAMP-dependent protein kinases and subsequent phosphorylative modification of substrate proteins (9), we have analyzed nuclear protein kinase activity during and following the second wave of increase of cAMP levels before the onset of DNA synthesis in the regenerating rat liver.

MATERIALS AND METHODS Partial hepatectomy was performed on male Sprague-Dawley rats (150-200 g) according to the procedure of Higgins and Anderson (10). Sham hepatectomy was performed in the same manner except for the ligation and removal of the liver lobes.

Nuclei were isolated in hypertonic sucrose as described by Yu (11), except that $MgCl_2$ was used instead of $CaCl_2$. In order to remove nonspecifically bound cytoplasmic cAMP-dependent protein kinase catalytic subunit, the nuclear pellet was washed in 10 mM imidazole acetate (pH 7.4), 4 mM $MgCl_2$, 0.5 mM phenylmethyl sulfonylfluoride, 5 mM mercaptoethanol (Buffer A) containing 0.32 M sucrose and 0.15 M NaCl according to the procedure of Keely et al. (12). The nuclei were recovered by centrifugation and resuspended in Buffer A containing 0.25 M sucrose. The number of nuclei in the suspension was determined in a Coulter Counter (Coulter Electronics, Hialeah, Florida) and aliquots were used for the determination of total nuclear protein kinase activity in unfractionated nuclei.

Kinetic analysis of nuclear protein kinase activity was carried out with nuclear 0.35 M NaCl extracts. These extracts were prepared by extraction of nuclei for 30 min with Buffer A containing 0.35 M NaCl. The insoluble nucleohistone was removed by centrifugation at 105,000 x g for 60 min. The NaCl concentration in the extracts was reduced by dilution with Buffer A to 0.15 M before assay of kinase activity.

For ion-exchange chromatography, the 0.35 M NaCl extracts were first subjected to gel filtration on Ultrogel AcA 34 (LKB Instruments, Inc., Chicago, Illinois) (13), followed by dialysis and fractionation of the extract on DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, New Jersey).

Protein kinase determination was carried out in a total incubation volume of 0.2 ml containing: 200 µg substrate protein; 25 mM imidazole acetate (pH 7.0); 10 mM magnesium acetate; 1 mM dithiothreitol; 2 mM theophylline; either 100 µM ATP (unfractionated nuclear protein kinase) or 2.5 µM ATP (protein kinase from DEAE-Sephacel elution); 1 µCi [^{32}P]ATP (>2500 Ci/mmol, Amersham Corporation, Arlington Heights, Illinois or ICN Pharmaceuticals, Irvine, California); without or with 10^{-7} M cAMP as described in the figure legends. Incubation was started by the addition of the enzyme source and carried out for 10 min at 35°C. The reaction was terminated by the addition of 2 ml of 25% trichloroacetic acid (TCA) containing 1% sodium dodecyl sulfate and 1 mM ATP. The samples were filtered using Millipore filters (0.3 µ). The filters were washed four times with 6 ml of 10% TCA, dissolved in 10 ml of scintillation cocktail and counted in a Packard Tri-Carb Scintillation Spectrometer.

Heat-stable inhibitor from rabbit skeletal muscle was isolated according to Ashby and Walsh (14). Protein was determined by the method of Bradford (15) using bovine gamma globulin as standard.

Significance was determined using the student's t test.

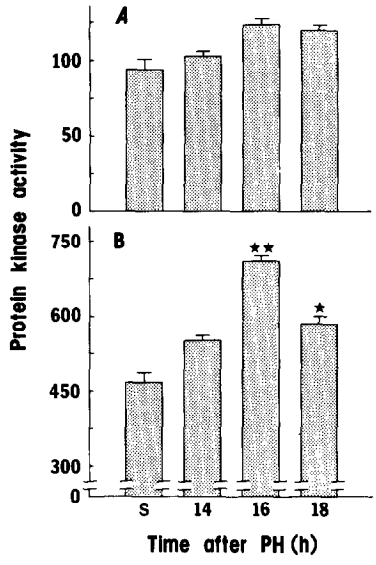

Fig. 1. Rat liver protein kinase activity in unfractionated nuclei after PH. Protein kinase activity is expressed as pmol ^{32}P incorporated/min/mg protein (A) or pmol ^{32}P incorporated/min/10^8 nuclei (B). Assays were done in the presence of 10^{-7} M cAMP as described in the Methods, with protamine sulfate as substrate. S=sham-operated controls; * = P<0.10 ** = P<0.05
Values are reported as the Mean ± S.E.M. from a minimum of three experiments.

RESULTS When protein kinase activity in unfractionated nuclei is assayed and expressed as ^{32}P incorporated per mg protein (Fig. 1A), a slight but not statistically significant increase of kinase activity is observed after PH. Analysis of the nuclear protein content shows a significant increase at 15-17 hours after PH (Table I). Since these changes in nuclear protein content obscure nuclear kinase activity changes, enzyme activity is also expressed on a per unit number

TABLE I

Nuclear protein content after partial hepatectomy

Time after PH (h)	mg protein / 10^8 nuclei	
Sham	4.88 ± 0.07	
14	5.42 ± 0.11	
15	5.68 ± 0.07	(P<0.025)
16	5.74 ± 0.05	(P<0.001)
17	5.62 ± 0.04	(P<0.025)
18	4.84 ± 0.05	

Values are reported as the Mean ± S.E.M. from a minimum of three experiments.

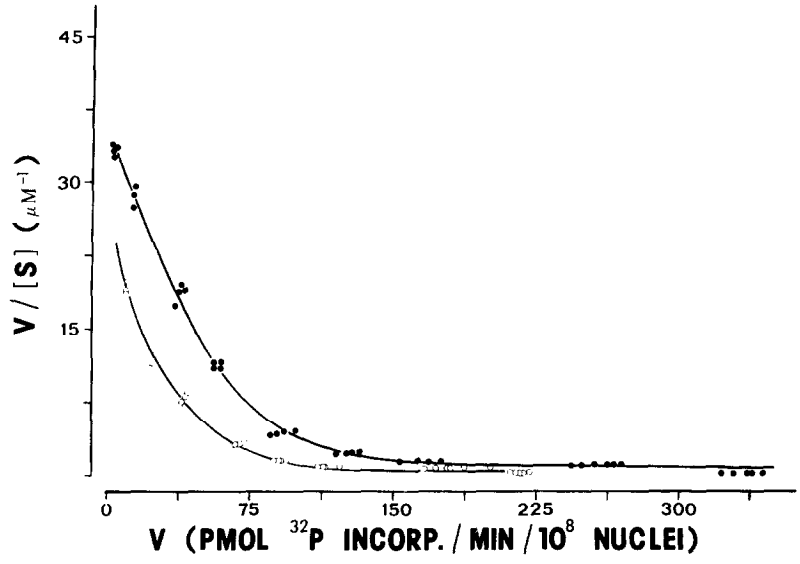

Figure 2. Kinetic analysis of nuclear protein kinase activity in 0.35 M NaCl extracts (Eadie-Scatchard plot). Protein kinase activity in nuclear 0.35 M NaCl extracts obtained from 16-hour regenerating livers (●—●) or from the livers of sham-operated controls (○—○) was determined in the presence of 10^{-7} M cAMP as described in the Methods, with protamine sulfate as substrate.

of nuclei. When plotted in this fashion, a significant rise of the specific activity of protein kinase is observed 16 and 18 hours after PH, with an activity peak at 16 hours (Fig. 1B).

Kinetic analysis of nuclear 0.35 M NaCl extracts using an Eadie-Scatchard plot (Fig. 2) reveals a significant increase ($P<0.05$) of kinase activity 16 h after PH at all ATP concentrations tested. Since the curvilinear behavior of the plot in Fig. 2 suggests the presence of multiple species of kinase, we proceeded to analyze the multiplicity of kinases by ion-exchange chromatography.

Nuclear 0.35 M NaCl extracts were first subjected to gel filtration to remove aggregating proteins which form upon dilution of the NaCl concentration. Subsequent fractionation on DEAE-Sephacel resolves nuclear protein kinase activity into four kinase species (Fig. 3). With total histone as substrate (Fig. 3, panels A and C) a cAMP-insensitive kinase activity elutes at 2.2 $mmho^{-1}$ (peak I) and a cAMP-dependent activity at 6.8 $mmho^{-1}$ (peak III). Using the heat-stable inhibitor from rabbit skeletal muscle, which specifically inhibits the catalytic subunit of cAMP-dependent protein kinase (14), it is found that

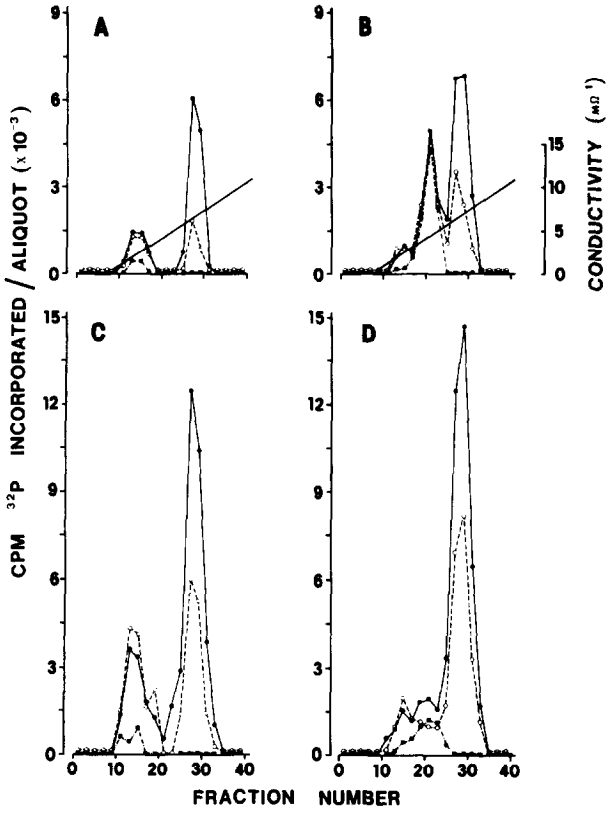

Figure 3. DEAE-Sephacel elution profiles of nuclear protein kinase activity in nuclear 0.35 M NaCl extracts. Fractionation of the 0.35 M NaCl extracts and determination of protein kinase activity were performed as described in the Methods. Protein kinase assays were performed in the absence (O---O) or presence (●—●) of 10^{-7} M cAMP using either total histone (type II-AS, Sigma Chemical Company, St. Louis, Missouri) (panels A and C) or protamine sulfate (panels B and D) as substrate. ■···■, activity determined in the presence of cAMP and saturating concentrations of the heat-stable protein kinase inhibitor. Panels A and B: sham-operated control liver; Panels C and D: 16-hour regenerating liver.

after sham-operation about 70% of the activity in peak I is inhibited by saturating concentrations of the inhibitor. This indicates the presence of two types of activity in peak I e.g., a cAMP-dependent (peak I_a) and a cAMP-independent activity (peak I_b). Peak III is completely inhibited indicating the presence of cAMP-dependent activity only. With protamine sulfate as substrate (Fig. 3, panels B and D) an additional cAMP-independent peak of activity (peak II) elutes at 4.5 mmho^{-1}.

DEAE-Sephacel analysis of nuclear protein extracts obtained 16 h after PH shows qualitatively identical elution profiles (Fig. 3, panels C and D) as

extracts from sham-operated controls (Fig. 3, panels A and B). Because equal amounts of protein were used on all DEAE-Sephacel columns, the peak heights can be used as a semi-quantitative estimate of relative kinase activity. Comparison of the elution profiles from several experiments indicates an approximately 5- to 8-fold increase of kinase activity in peak I_a and a 2- to 4-fold increase of kinase activity in peak III. The activity of peak II is reduced after PH.

DISCUSSION These data provide evidence for the presence of multiple cAMP-independent and cAMP-dependent protein kinase species in the nuclei of control and regenerating liver. During the prereplicative phase examined, nuclear cAMP-dependent as well as -independent protein kinases undergo marked activity changes. Siebert et al. (16), Whitfield et al. (17), and Chen et al. (18) have previously reported similar activity changes of total nuclear protein kinase activity but have not analyzed the multiplicity of nuclear kinases. Additional findings suggest that the nuclear protein kinase modulation is manifested by the selective phosphorylation of histones (18-20) and nonhistone chromosomal proteins (21) at times which correlate well with the times of increased nuclear protein kinase activity reported by us. The precise role of cAMP in the modulation of nuclear protein kinase species and the potential significance of nuclear protein kinase activity changes during rat liver regeneration are presently being investigated in our laboratory.

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