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PHOSPHORYLATION OF A NONHISTONE PROTEIN FRACTION WHICH COEXTRACTS WITH THE HIGH-MOBILITY-GROUP PROTEINS OF CHROMATIN

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Summary: ^{32}P-labelled chromatin proteins from rat liver and ventral prostate were fractionated according to the procedure designed to enrich high-mobility-group (HMG) nonhistone proteins. This fraction, however, reproducibly demonstrated small amounts of apparently basic nonhistone proteins other than HMG nonhistone proteins. These proteins appeared to be tissue specific and were highly labelled with ^{32}P. The ^{32}P-labelled phosphoproteins were soluble in trichloroacetic or perchloric acid, migrated in acid-urea polyacrylamide gels, and demonstrated pI values ranging from 6.8 to 7.5. The HMG proteins 1 and 2 showed no incorporation of radioactivity under these experimental conditions.

Chromatin is comprised of a variety of chromosomal proteins including histones, nonhistones, and the products of their post-synthetic modifications (1-5). These proteins continue to be the subject of much investigation because of their possible role in chromatin structure and function. A variety of nonhistone proteins bound to DNA are extracted with 0.35 M NaCl. They can be further fractionated according to their solubility in trichloroacetic acid. The soluble fraction obtained after the latter manipulation contains the so called "high-mobility-group" (or HMG) proteins which are nonhistone proteins but are basic in nature (6,7). In an attempt to study the phosphorylation of various proteins in this fraction, we observed that a number of proteins were highly labelled with ^{32}P. However, most of the radioactivity was present in faintly stained proteins which had electrophoretic mobility in acid-urea polyacrylamide gels lower than that of HMG proteins. Their apparent acid solubility, electrophoretic behavior, and pI values suggest that these nonhistone phosphoproteins are not acidic in nature and may be relatively basic, although they may not belong to the same class as the HMG proteins. They

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did not seem to arise from degradation of other proteins, and their presence was highly reproducible. Further, the pattern of ^{32}P incorporation in these proteins showed some tissue specificity.

Experimental Procedures

Preparation of chromatin and ^{32}P-labelled proteins. Rat ventral prostate and liver chromatin preparations were obtained as described previously (8,9). Chromatin-associated proteins were labelled with ^{32}P in a reaction medium consisting of 30 mM Tris-HCl, pH 7.45 (at 37°C), 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM [γ-^{32}P]-ATP ($\sim$ 1 x 10^6 dpm/nmol of ATP), and an equivalent of 1.5 to 3 mg of chromatin protein, in a final reaction volume of 5.0 ml. The reaction was carried out for 30 min at 37°C to achieve maximal labelling of various proteins (9). A suitable volume of ice-cold 2 M NaCl was then added to the reaction so as to achieve a final concentration of 0.35 M with respect to NaCl. This mixture was stirred on ice for 60 min and then centrifuged for 10 min at 10,000 x g. The pellet was similarly re-extracted, and the extracts were pooled. The fraction containing HMG proteins was isolated according to the procedure of Goodwin et al. (10). Briefly, the extract was made 2% with respect to trichloro-acetic acid (w/v) and then centrifuged for 20 min at 20,000 x g. To the supernatant phase was added 2-mercaptoethanol to a final concentration of 10 mM, and the pH was adjusted to 10.0. To this mixture, three volumes of acetone were added, and after allowing it to stand for 24 hr at -20°C, it was centrifuged for 15 min at 20,000 x g. The pellet so obtained was washed twice with cold acetone/0.1 N HCl (6:1, v/v) followed by cold acetone three times. The pellet was dried under vacuum and then dissolved in a suitable volume of 0.9 N acetic acid/6.0 M urea/15% sucrose solution to carry out electrophoretic separation as described by Panyim and Chalkley (11). The electrophoresis was carried out for 3 hr at 1.7 mA/gel, after which the gels were stained directly using Coomassie brilliant blue-G-250 stain. The stained gels were scanned on an Ortec densitometer. Duplicate gels were fixed in 20% trichloroacetic acid and sliced into 1 mm sections using a Mickle gel slicer. The slices were placed in a liquid scintillation medium (Omnifluor from New England Nuclear) and the radioactivity was measured using a Packard Tricarb liquid scintillation spectrometer.

The fraction enriched in HMG proteins was also prepared by extracting ^{32}P-labelled nuclei with 0.74 M perchloric acid according to Sanders and Johns (12). These proteins were then precipitated with 20% trichloroacetic acid. The residue was washed with acetone/0.1 N HCl (6:1, v/v), followed by cold acetone alone. The dried pellet was dissolved in 50 mM sodium phosphate buffer, pH 6.5 (20°C) containing 4% guanidine·HCl, and fractionated on Bio Rex 70 column as described by Seyedin and Kistler (13).

Analytical isoelectric focusing in polyacrylamide gel was based on the procedure of O'Farrell (14) with the modification that NP-40 was omitted and riboflavin/persulfate TEMED were used to polymerize the gels. The ampholyte concentration was 3%, and its pH range was 3.0 to 10.0. (Ampholyte was obtained from Bio-Rad, Richmond, CA).

Results and Discussion

Figs. 1.A1 and 1.B1 show a comparison of the polyacrylamide gel electrophoretic behavior of various proteins in the fraction enriched for HMG proteins, isolated from rat liver and ventral prostate tissues. Some difference in the electrophoretic profile of the proteins with low mobility in the two samples was apparent. When purified chromatin preparations from rat liver or ventral prostate

were reacted with [γ-^{32}P]-ATP, followed by fractionation of loosely bound nonhistone proteins according to the procedure of Goodwin et al. (10), we observed that a number of proteins in this group were phosphorylated as judged by the incorporation of ^{32}P. Figs. 1.A2 and 1.B2 show gel electrophoretic profiles for [^{32}P]-labelled proteins from rat liver and ventral prostate. Most of the radioactivity was present in proteins with mobilities lower than the HMG proteins. These highly labelled proteins represented only a small quantity of the total proteins in this fraction. The amount of radioactivity incorporated into these proteins differed with the tissue, e.g., in the present case, ventral prostate proteins appeared to be more actively phosphorylated than those from liver. Also, compared with the total 0.35 M NaCl-soluble fraction, a large portion of the radioactivity per mg of protein was present in this acid soluble fraction. The proteins migrating on gels to the area corresponding in mobility to the HMG and histone proteins had relatively little radioactivity (Figs. 1.A2 and 1.B2). It should also be mentioned that the profiles of phosphorylation of the nonhistone proteins shown in Figs. 1.A2 and 1.B2 are not likely to be due to the presence of minute quantitites of highly labelled histones since the latter are poorly phosphorylated under the experimental conditions used (8,9,15) and are largely eliminated in the general preparative procedures used in this study.

When fractionation of the various proteins was carried out by Bio-Rex 70 column chromatography (13), the aforementioned highly radioactive proteins of lower mobility were recovered mostly in the flow through peak suggesting that they are not as basic as the HMG proteins or histones. That these nonhistone proteins are not acidic in nature is further supported by the fact that they were originally extracted in 0.74 M perchloric acid, as described under Experimental Procedures. Thus, the evidence provided above strongly suggests that these chromatin-associated nonhistone proteins which are capable of undergoing a high degree of phosphorylation are basic in nature. The apparent molecular weights of these basic nonhistone proteins, as determined by SDS-gel electrophoresis, were between 14 to 70 K (results not shown). HMG 1 and 2 were eluted from Bio-Rex 70 column (13) and identified by gel electrophoresis

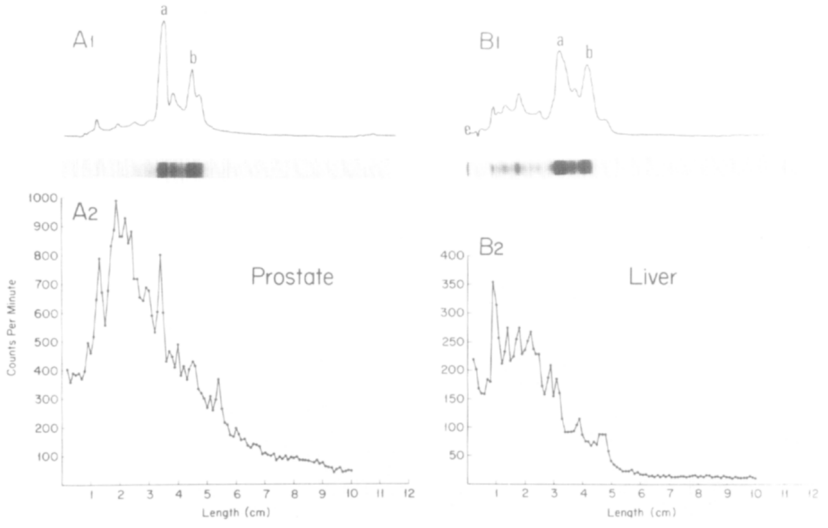

Fig. 1: The acid urea polyacrylamide gel electrophoretic pattern of rat ventral prostate (A) and liver (B) nonhistone protein fraction prepared by the method in (10). A1 and B1 represent gels stained for protein and their respective densitometer tracing. 44.1 µg of protein from liver, and 26.0 µg of protein from prostate nonhistone protein fractions were applied to each gel. Electrophoresis is from left to right. In Figs. A1 and B1, the region between "a" and "b" corresponds to where HMG 1,2, histone and HMG 14,17 migrate. A2 and B2 depict the profile of ^{32}P radioactivity distributed in various proteins, and was determined from duplicate gels corresponding to those presented in Figs. A1 and B1. The radioactivity in various proteins shown in Figs. A2 and B2 was normalized to the same concentration of protein for each tissue. The specific radioactivity of [γ-^{32}P]-ATP was the same in each experiment.

against known standard samples of the same proteins. No measurable radioactivity was detected in the two proteins. Further characterization of the ^{32}P-labelled phosphoproteins of the flow through fraction was carried out by analytical isoelectric focusing as shown in Fig. 2. It was observed that only a small amount of radioactivity was present in proteins with pI ranging from 5.6 to 6.5, and from 7.5 to 8.0. The fraction which focused in the pI range of 6.8 to 7.5 contained the most highly radioactive proteins. Since the pI values obtained were for the phosphorylated nonhistone proteins, it can be presumed that their dephospho forms are likely to be more basic in nature.

The differences in levels of phosphorylation of basic nonhistone proteins in liver and prostate described above may reflect tissue-specificity. It is also plausible that this is due to different amounts of various phosphoproteins, or to variations in the protein kinases present in chromatin, since some tissue-specific

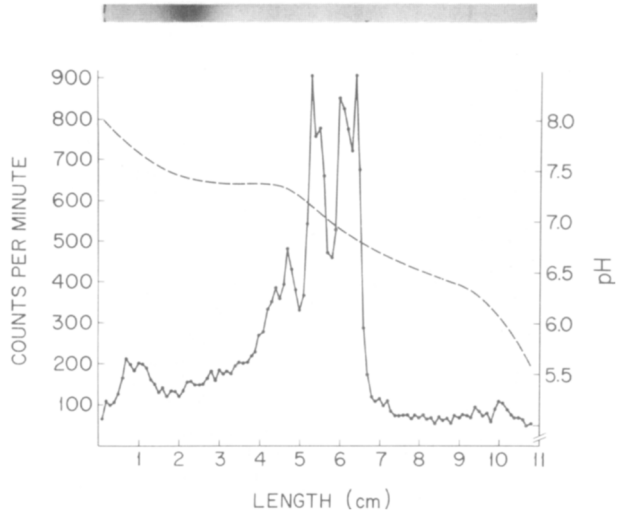

Fig. 2: Analytical isoelectric focusing in polyacrylamide gel of rat liver ^{32}P-labelled nonhistone protein fraction prepared by methods in (12,13).

heterogeneity with respect to these enzymes has been observed by us for rat liver and ventral prostate (unpublished results). That the above results were not artifacts due to the presence of proteolytic activity, endogenous protein phosphatase activity, or differences in the metabolic state of the organs was established by the following additional observations: first, a time course study of ^{32}P incorporation into various proteins was carried out and samples were analyzed at 10, 30 and 60 min. The labelling in all proteins under the given experimental conditions remained essentially constant between 15 and 60 min. Second, addition of 0.5 mM phenylmethylsulfonyl fluoride (a potent protease inhibitor) or 10 mM NaF (a phosphtase inhibitor) did not alter the electrophoretic pattern of ^{32}P labelling of the various proteins studied over a time course of up to 30 min. Third, the difference in the electrophoretic profile of ^{32}P labelling of the basic nonhistone proteins in the two tissues was highly reproducible, and was confirmed in twelve different experiments. Further, different preparative techniques described under Experimental Procedures yielded similar results.

It is generally believed that basic nonhistone proteins of the HMG class show no tissue or species variation (6,7). However, as discussed by Weber and Isenberg (16), at present it cannot be determined whether or not the minor protein components

of the 0.35 M NaCl extractable, 2% trichloroacetic acid soluble protein class belong to the HMG nonhistone proteins. Some variation in HMG nonhistone proteins may be produced by post-synthetic modification such as by acetylation (17) of HMG 1, or phosphorylation (18) of HMG 14, 17. If the minor protein components of the HMG enriched fraction turn out to belong to this class of proteins, it may be that their phosphorylation (as shown here) reflects a means of generating tissue heterogeneity among these proteins. Be as it may, the apparent tissue variation of ^{32}P incorporation in the basic nonhistone phosphoproteins described in this work is in accord with the observations that chromosomal nonhistone proteins and their phosphorylated derivatives, in general, show tissue- and species-specificity (1-5). It has been documented that chromatin-associated nonhistone proteins extracted in 0.35 M NaCl show tissue variations in their composition (19) and phosphorylation (20). However, the present study has demonstrated that fractionation of 0.35 M NaCl-soluble nonhistone proteins (using trichloroacetic acid) to obtain the HMG proteins also yields a group of apparently basic, tissue-specific nonhistone proteins whose mobility in acid-urea polyacrylamide gels is lower than that of HMG proteins. These proteins are capable of being extensively phosphorylated by protein kinase reactions intrinsic to chromatin. Some of these proteins and phosphoproteins may have been detected in other work which utilized H_2SO_4 as the extracting agent (21,22). Besides the basic nonhistone proteins described herein, several investigators have recently reported the presence of other basic nonhistone proteins (different from HMG proteins); however, phosphorylation of these proteins was not investigated (23-25). It would be of interest to determine a role of the highly phosphorylated basic nonhistone proteins described in the present work, especially in regard to chromosomal structure/function.

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