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PHOSPHORYLATION AND ACETYLATION OF CHROMATIN CONJUGATE PROTEIN A24

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SUMMARY

An analysis was made of the phosphorylation and acetylation of chromatin protein A24, a conjugate of histone 2A and ubiquitin. ^{32}P-orthophosphate was incorporated into phosphoserine of histone 2A and protein A24 in Novikoff hepatoma ascites cells in culture. The ratio of ^{32}P incorporation and the pattern of tryptic digestion of ^{32}P-labeled protein A24 indicated that the histone 2A component was phosphorylated and the ubiquitin component was not. Analysis of ε-N-acetyl lysine in protein A24, histone 2A and ubiquitin showed that while protein A24 and histone 2A were acetylated, ubiquitin was not. Apparently, even though it is conjugated with ubiquitin, the histone 2A portion of protein A24 has the same modifications as free histone 2A. The lack of modification of ubiquitin differs from that of high mobility group (HMG) nonhistone chromatin proteins with which it is co-extracted from chromatin.

INTRODUCTION

The amino acid sequence of proteins is defined at the level of transcription and translation, but post-synthetic modifications amplify the multiplicity of the amino acids in proteins from the 20 specified by the genetic code to approximately 140 that are found in nature (1). In the case of histones, two of these amino acid modifications, the phosphorylation of serines and threonines (2) and the ε-N-acetylation of lysines (3,4) have been correlated with cell replication and gene activation (5-9). The phosphorylation of nonhistone chromatin proteins has also been correlated with gene activation (10-12). Recently, the high mobility group (HMG) nonhistone chromatin proteins (13) have been shown to contain

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ε-N-acetyl lysine (14) which may be involved with their functions as DNA binding proteins (15-17) associated with actively transcribing chromatin (18,19).

Chromatin conjugate-protein A24 has a branched structure (19) composed of histone 2A (21) and ubiquitin (22-24), the carboxyl terminal amino acid of which is attached through glycylglycine to the ε-NH_2 of lysine 119 of histone 2A (25). Inasmuch as free histone 2A undergoes phosphorylation and acetylation (7-9) and free ubiquitin has been found among the HMG proteins (26-28), the present study of the phosphorylation and acetylation of protein A24 was initiated to determine whether protein A24 has similar modifications.

MATERIALS AND METHODS

Purification of Proteins. Protein A24 was purified from calf thymus chromatin by differential extraction, gel filtration and preparative polyacrylamide gel electrophoresis as previously described (29). Protein A24 labeled with ^{32}P-orthophosphate was purified in the same manner from Novikoff hepatoma chromatin prelabeled as described below. Histone 2A was purified from calf thymus chromatin as described previously (30). Calf thymus ubiquitin was the generous gift of Dr. Goldstein of Ortho and was purified by a previously described procedure (31).

Analysis of Phosphorylation. Novikoff hepatoma cells which had been grown in Holtzman male albino rats were harvested and washed 2 times in 0.13 M NaCl, 5 mM KCl, 8 mM $MgCl_2$ followed by incubation for 2 hours at 37° in media containing 100 mCi of carrier-free ^{32}P orthophosphate (New England Nuclear). Citric acid nuclei (32) were then prepared and chromatin made from them by a modification (33) of the method of Marushige and Bonner (34). Acid soluble proteins were extracted twice with 0.4 N H_2SO_4. The acetone precipitated acid extracts were subjected to two-dimensional polyacrylamide gel electrophoresis by the method of Orrick *et al* (35) and ^{32}P incorporation into the proteins monitored by autoradiography and scintillation counting (12). For phosphoserine and phosphothreonine analysis of ^{32}P labeled purified protein A24, samples were subjected to partial hydrolysis in 2 N HCl at 110° *in vacuo* for four and eight hours. The hydrolysates were analyzed by paper electrophoresis in 2% formic acid, 8% acetic acid, pH 1.8 for 90 minutes at 3000 V, followed by autoradiography. To determine whether ^{32}P-orthophosphate was incorporated into the ubiquitin portion of protein A24, samples were treated with 1% weight ratio of trypsin twice at 37° for 2 hours each in 0.1 M N-ethylmorpholine acetate pH 8.0. The digested and undigested samples were subjected to electrophoresis in 15% polyacrylamide gel slabs (Hoeffer Apparatus, 1 mm thick) according to

the method of Laemmli (36). Distribution of ^{32}P-orthophosphate was monitored by autoradiography and distribution of protein by Coomassie brilliant blue R (Sigma) stain.

Analysis of ε-N-Acetyl Lysine. Purified protein A24, histone 2A and ubiquitin were analyzed for the presence of ε-N-acetyl lysine by the method of Sterner et al (14). Samples were subjected to digestion with a 1% weight ratio of trypsin twice for two hours at 37° in 0.2 M N-ethylmorpholine acetate, pH 8.1. After placing the samples in a boiling water bath for 5 minutes they were subjected to digestion with a 10% weight ratio of pronase for 18 hours at 40°. The samples were then vacuum-dried and released amino acids analyzed on a Beckman 121MB amino acid analyzer. The retention time of ε-N-acetyl lysine was determined using an external standard of ε-N-acetyl lysine (Aldrich).

RESULTS

Novikoff hepatoma ascites cells were incubated in vitro. The pattern of incorporation of ^{32}P-orthophosphate into 0.4 N H_2SO_4 soluble chromatin proteins is shown in Figure 1. Among those proteins labeled were protein A24 and histone 2A.

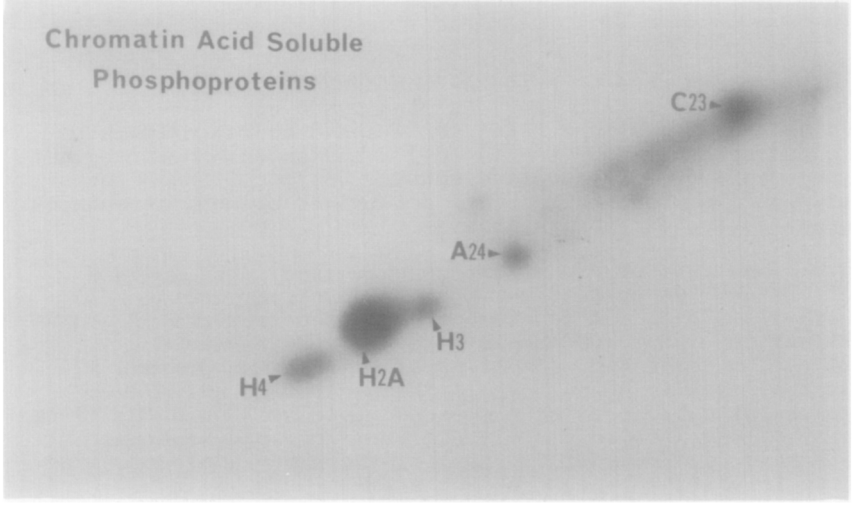
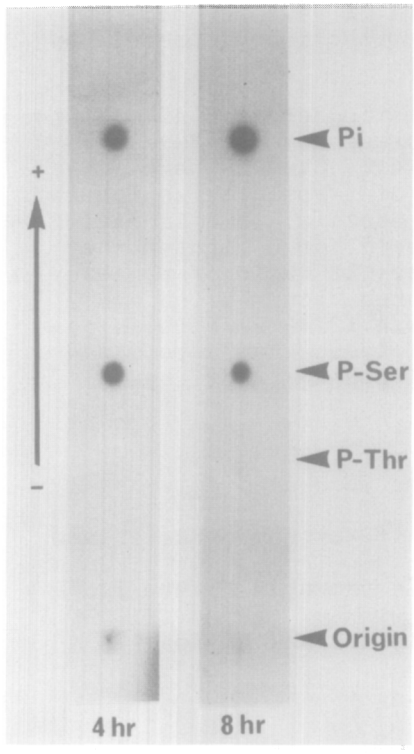
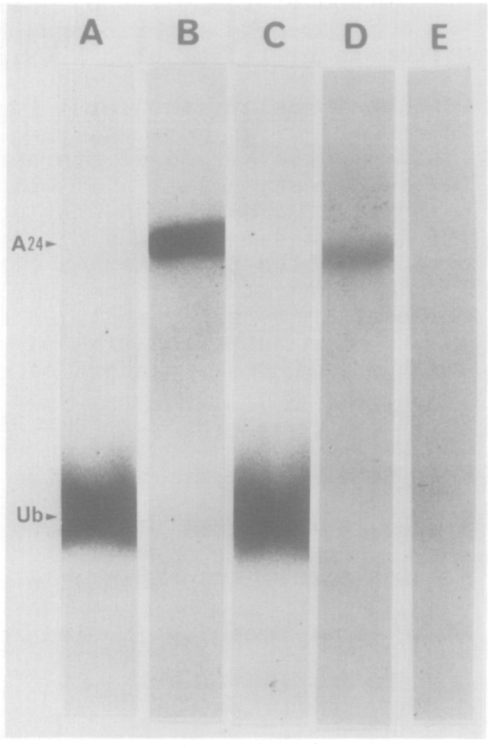


Figure 1. Analysis of 0.4 N H_2SO_4 soluble chromatin phosphoproteins by two-dimensional polyacrylamide gel electrophoresis. The Novikoff hepatoma chromatin acid extract (500 µg, 139,000 CPM) dissolved in 10 M urea, 0.9 N acetic acid, 1% 2-mercaptoethanol was subjected to electrophoresis from right to left in 10% polyacrylamide, 0.9 N acetic acid, 4 M urea gel. The second dimension from top to bottom was 12%, polyacrylamide, 0.1% sodium dodecylsulfate, 0.1 M sodium phosphate, 6 M urea, pH 7.1. Autoradiography was for 9 days. The identification of radioactive protein was done in comparison to Coomassie blue stained protein spots and the known mobilities of these proteins.



(2)



(3)

Figure 2. Analysis of the incorporation of ^{32}P-orthophosphate into phosphoserine and phosphothreonine residues of purified ^{32}P labeled Novikoff hepatoma protein A24. Samples (1,000 cpm) were subjected to partial hydrolysis in 2 N HCl for 4 and 8 hours followed by paper electrophoresis (bottom to top). The autoradiographs indicate the position of radioactive spots. The positions of phosphoserine and phsophotheonine were determined by coelectrophoresis of nonradioactive external standards.

Figure 3. Analysis of ^{32}P orthophosphate incorporation into protein A24 and its ubiquitin component. Polyacrylamide gel electrophoresis was from top to bottom in the presence of sodium dodecylsulfate (36). Coomassie blue stained gels were (A) 10 µg of calf thymus ubiquitin, (B) 10 µg of calf thymus protein A24, (C) 20 µg of tryptic digested calf thymus protein A24. Autoradiographs of corresponding electrophoretic gels were (D) 33 µg (500 cpm) of ^{32}P labeled protein A24 from Novikoff hepatoma chromatin and (E) 66 µg (1,000 cpm) of the same sample digested with trypsin prior to electrophoresis.

The ratio of incorporation of ^{32}P into histone 2A and protein A24 was 10.6 to 1 which closely approximates the 10 to 1 molar ratio of these two proteins (29,37). Protein A24 labeled with ^{32}P was purified from the extracts as described previously (29) and subjected to partial acid hydrolysis. As shown in Figure 2, at

both 4 and 8 hours of hydrolysis radioactivity comigrated predominantly with phosphoserine and inorganic phosphate. Similar results were obtained with ^{32}P labeled histone 2A purified from the same extracts.

It has been previously shown that ubiquitin is resistant to tryptic cleavage (24) and is obtained intact upon treatment of protein A24 with trypsin (9). When ^{32}P labeled protein A24 was treated with trypsin (Fig. 3) no radioactivity was detected in the ubiquitin moiety. Thus phosphorylation of protein A24 is apparently only on the histone 2A part of the protein A24 molecule. In fact, preliminary studies indicate that the phosphopeptide maps of protein A24 and histone 2A are essentially identical.

To study ε-N-acetylation of lysines, the trypsin-pronase method of Sterner et al (14) was used with purified protein A24, histone 2A and ubiquitin (Fig. 4). In the digests of protein A24 and histone 2A, ε-N-acetyl lysine was detected, but in the digest of ubiquitin, no ε-N-acetyl lysine was detected. Thus, the ε-N-acetyl lysine detected in the analysis of protein A24 probably arises from the histone 2A part of the molecule. Ubiquitin was apparently unacetylated.

DISCUSSION

This study shows that the histone 2A portion of protein A24 has the same modifications as free histone 2A. Furthermore, the phosphorylation and acetylation of protein A24 is contributed by the phosphorylation and acetylation of histone 2A alone. The same may be the case for poly ADP ribosylation of protein A24 inasmuch as studies of Okayama and Hayaishi (38) show a ratio of in vitro poly ADP ribosylation of histone 2A and protein A24 of 10 to 1, consistent with modification of the histone 2A moiety alone.

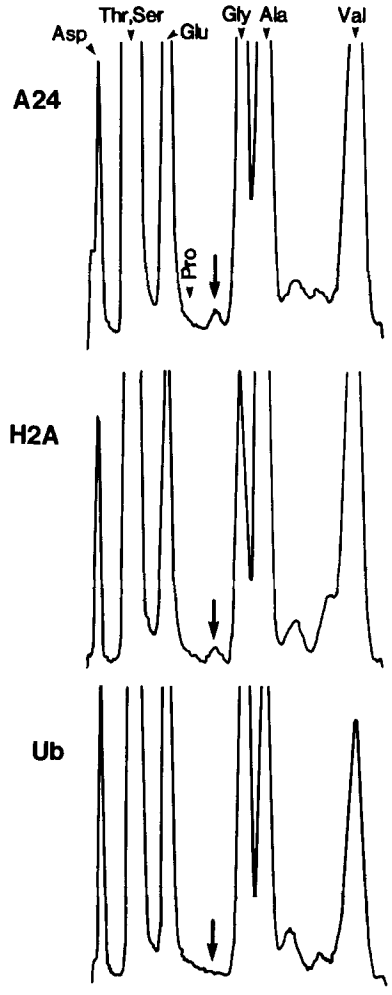


Figure 4. Analysis of ε-N-actyl lysine in protein A24, histone 2A and ubiquitin. Purified protein samples from calf thymus were subjected to trypsin-pronase treatment as in methods. Aliquots of the digest were then dried and subjected to analysis of amino acids released using a Beckman model 121MB amino acid analyzer. The pattern of elution of aspartic acid through valine is shown and the position of ε-N-acetyl lysine detected in protein A24 (top) and histone 2A (middle) but not in ubiquitin (bottom) is denoted by large arrow between the positions of proline and glycine.

The lack of modification of ubiquitin is somewhat puzzling inasmuch as the high mobility group (HMG) nonhistone chromosomal proteins which are co-isolated with free ubiquitin (26,27) have been shown to be acetylated (14) and poly ADP ribosylated (39). However, while HMG proteins have been reported to bind DNA (15-17), this is not true of ubiquitin (40).

It is now generally accepted that histones 2A, 2B, 3 and 4 are organized into an octamer which forms the protein core around which the chromatin DNA is wrapped to form the nucleosome subunit structure (41-43). Approximately 10% of nucleosomes contain protein A24, replacing unconjugated histone 2A in the core octamers (44,37,45). Levels of protein A24 were markedly diminished in transcriptionally enhanced hypertrophic nucleoli (46) and in transcriptionally active chromatin fractions (28). Moreover, free ubiquitin has been co-isolated with transcriptionally active chromatin fractions (27,28). This suggests that cleavage of the ubiquitin-histone 2A bond of protein A24 may be involved in transcriptional activation of chromatin by producing a conformational change in the template (47).

However, the presence of post-synthetic modifications of histone 2A moiety of protein A24 implies that the conjugated protein structure is stable to other local conformational changes in chromatin. These include changes occurring during the deposition of histones and replication of DNA which is correlated with phosphorylation and acetylation of histone 2A (7-9). In addition, DNA repair may be related to poly ADP ribosylation (48,49), during which the protein A24 structure is also stable.

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