Postsynthetic Modification of High Mobility Group Proteins

EVIDENCE THAT HIGH MOBILITY GROUP PROTEINS ARE ACETYLATED*

(Received for publication, August 3, 1978, and in revised form, August 29, 1978)

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SUMMARY

High mobility group proteins were isolated from calf thymus and duck erythrocyte nuclei and the possibility was investigated that these proteins undergo acetylation similar to that occurring in some histones.

Dinitrophenylation of the proteins followed by acid hydrolysis and amino acid analysis indicated that 2 to 3% of the lysine residues present were unavailable for reaction with fluorodinitrobenzene. Extensive enzymatic degradation with trypsin and pronase and subsequent amino acid analysis showed a significant amount of material eluting at the position of \( \text{e-N-acetyllysine} \). Recovery and acid hydrolysis of this material generated a peak eluting in the lysine position. In vitro radioactive labeling of calf thymus nuclei with \( ^{3} \text{H} \)acetate yielded labeled high mobility group proteins.

All of these findings are in accord with the conclusion that high mobility group proteins are acetylated and that acetylation occurs as a postsynthetic modification of these proteins.

The phenomenon of nuclear protein acetylation has been a field of intensive study ever since the first discoveries of two forms of such modification in histones (1, 2). With the observations that acetylation of lysine residues in histones occurs as a postsynthetic process (2-4) came the implication of histone acetylation as a possible means of control of chromatin structure or function, or both. In fact, much evidence has been obtained suggesting a correlation between gene activity and degree of acetylation (2-9) as well as a correlation between the degree of acetylation and the effectiveness of histones in producing distortions of DNA conformation (10). The present report deals with similar modification of other chromosomal proteins.

A group of non-histone chromosomal proteins can be obtained from the chromatin of various tissues by extraction with 0.35 M NaCl. These proteins can be fractionated into two main groups by differential precipitation with trichloroacetic acid. These groups were named on the basis of their respective electrophoretic mobilities in 20% polyacrylamide gels at pH 2.4. The proteins with low electrophoretic mobilities were called low mobility group proteins and those with high electrophoretic mobilities were named high mobility group proteins (11, 12). It is the latter group that is the subject of this investigation.

HMG proteins, like the histones, have been extracted from isolated nucleosomes (13, 14) and thus have been implicated as possibly having some role in chromatin structure or function, or both. It has been demonstrated that the HMG-1 and HMG-2 from calf thymus readily bind to DNA (15-17) as well as to histone H1 (15, 16). Furthermore, limited digestions of chromatin with deoxyribonuclease I under conditions which have been shown to selectively destroy active or formerly active genes (19-21) causes the release of HMG proteins (14, 21), and reconstitution experiments with chicken erythrocyte chromatin produced evidence suggesting that HMG proteins enhance transcriptional activity (22).

Given that HMG proteins do appear to be intimately associated with chromatin and that they have DNA-binding properties, it seemed possible that they undergo some of the same types of postsynthetic structural modifications (e.g., acetylation) associated with histones. It is this possibility that we set out to investigate. In particular, attention was focused on the two major HMG proteins from calf thymus (HMG-1 and HMG-2) and the two major proteins from duck erythrocytes (HMG-1 and HMG-E (23)). In the course of our experiments, we established that these proteins exist in acetylated form. This represents the first evidence for postsynthetic modification of the HMG protein class.

EXPERIMENTAL PROCEDURES

Dinitrophenylation—The procedure used was based on original methods described by Sanger (24) with incorporation of our own modifications as well as those of Wobsy and Singer (25) and Schroeder and LeGette (26). Protein (2 to 3 mg) was dissolved in 1 ml of NaHCO₃ solution (20 mg/ml). Following the addition of 2 ml of absolute ethanol, the denatured protein was shaken for 1 h. At this time, 30 \( \mu l \) of fluorodinitrobenzene were added and the mixture was shaken for 2 h at room temperature. The insoluble yellow product was washed twice each with water, ethanol, and ether successively and then hydrolyzed for 24 h in 4 N HCl at 110°C.

Enzymatic Degradation—A modification of the method of Gerseby et al. (27) was used. Protein (2 to 4 mg) was dissolved in 1 ml of 0.2 M N-ethylmorpholine acetate, pH 8.1. Trypsin (1% by weight of substrate) was added and the solution was incubated for 2 h at 37°C. At this time, an aliquot of fresh trypsin (1% by weight) was added and the hydrolysis was continued for another 2 h. The solution was placed in a boiling water bath for 5 min and allowed to cool. Pronase (10% by weight) was added and the reaction mixture was incubated for 18 h at 40°C and vacuum-dried prior to amino acid analysis.

Radioactive Labeling—Approximately 100 g of fresh calf thymus tissue was homogenized in incubation medium consisting of 200 ml of 0.32 M sucrose, 4 mM MgCl₂·6H₂O; 100 ml of 0.1 M sodium phosphate buffer, pH 6.8, in 0.25 M sucrose; and 80 ml of 0.1 M glucose containing 3.75 mg of NaCl and 4.2 mg of MgCl₂·6H₂O/ml. Following the addition of 25 ml of 1 M methyl-[\( ^{3} \text{H} \)acetylacetate, the homogenate was shaken for 30 min at 37°C. The nuclei were subsequently washed and the proteins were extracted as previously described (23).

Miscellaneous Procedures—Protocols employed in the isolation of nuclei, extraction and purification of HMG proteins, gel electropho-

* This work was supported by Grant 1-440 from the National Foundation March of Dimes, Grant CM 17383 from the National Institutes of Health, Grant NP-228H from the American Cancer Society, and by the Rockefeller Foundation Program in Reproductive Biology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

1 The abbreviation used is HMG, high mobility group.
Table 1

The extent of acetylation of lysine residues in some nuclear proteins as measured by lack of reactivity toward fluorodinitrobenzene

<table>
<thead>
<tr>
<th>Protein</th>
<th>% total lysine remaining as free lysine*</th>
<th>Moles acetylated lysine per mole protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus histone H1</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Calf thymus HMG-1</td>
<td>2.79</td>
<td>1.30</td>
</tr>
<tr>
<td>Calf thymus HMG-2</td>
<td>2.00</td>
<td>0.80</td>
</tr>
<tr>
<td>Duck erythrocyte HMG-1</td>
<td>2.82</td>
<td>1.18</td>
</tr>
<tr>
<td>Duck erythrocyte HMG-E</td>
<td>2.21</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*Measured by amino acid analysis following dinitrophenylation and acid hydrolysis (cf. "Experimental Procedures").

FIG. 1. Amino acid analysis of dinitrophenylated, acid-hydrolyzed calf thymus histone H1 (0.5 mg) and duck erythrocyte HMG-E (1.5 mg). The solid arrow designates the peak of free lysine remaining after such treatment, and the dashed arrow indicates the elution position of methyllysine (if it had been present). The broad peak eluting just following arginine (between 130 and 140 min) is e-N-dinitrophenyllysine. The analysis was performed under the standard conditions (pH 5.28, 55°C, buffer flow rate of 70 ml/h) using a column 15.5 cm long.

RESULTS

Dinitrophenylation of HMG Proteins—Representative chromatograms from amino acid analyses of acid-hydrolyzed, dinitrophenylated calf thymus histone H1 and duck erythrocyte HMG-E are compared in Fig. 1. H1, a "lysine-rich" histone, contains approximately 28 mol % lysine residues, none of which are acetylated (28), and the amount of residual free lysine following dinitrophenylation is 0.1% of the total lysine found in the native protein. Thus, the dinitrophenylation reaction was shown to be virtually quantitative for free e-amino groups. The chromatogram shown in the lower panel of Fig. 1 for HMG-E is typical of that found for all of the dinitrophenylated HMG proteins. The amount of free lysine was consistently found to be 2 to 3%, which corresponds approximately to 1 mol of lysine/mol of protein, based on a molecular weight of 26,000 for the calf thymus proteins (15, 16) as well as the duck erythrocyte HMGs.2 The relatively high amount of free lysine found in acid hydrolysates of dinitrophenylated HMG proteins suggests that a significant amount of lysine in these proteins exists in an acetylated or otherwise modified form. It should be mentioned here that methylation, known to occur in histones (29-32), and the other likely candidate for modification of lysine residues in HMG proteins, does not appear to be a process affecting these proteins. No peak corresponding to mono- or dimethyllysine has been discerned in acid hydrolysates of HMG proteins (data not shown). Dinitrophenylation, acid hydrolysis, and amino acid analysis of calf thymus histone H4, a histone known to undergo methylation (29, 31), clearly show a peak of dimethyllysine immediately following lysine.3 Examination


FIG. 2. Amino acid analysis of trypsin + pronase digests of calf thymus histone H1 and duck erythrocyte HMG-E (cf. "Experimental Procedures"). The arrows denote the elution position of e-N-acetyllysine.

Materials—Calf thymus glands were purchased from Max Cohen, Newark, N. J. Duck blood was kindly donated by Long Island Duck, Eastport, N. Y. Trypsin and pronase were purchased from Worthington and Calbiochem, respectively. Ninhydrin, N-ethylmorpholine, and fluorodinitrobenzene were products of Pierce Chemical Co., Rockford, Ill. All other reagents were of the purest commercially available grade.

of Fig. 1 reveals no such peak for HMG-E, and the peak was not observed for any of the other dinitrophenylated HMG proteins investigated. In Table I is presented a brief summary of the extent to which various nuclear proteins are acetylated as measured by the amount of free lysine present following dinitrophenylation and acid hydrolysis of the proteins.

Enzymatic Degradation of HMG Proteins—To further investigate the possibility of lysine modification and to more firmly identify the modifying group, extensive enzymatic degradation of the proteins with trypsin and pronase was employed. After enzymatic digestion, the samples were vacuum-dried and subjected directly to amino acid analysis. Fig. 2 shows the results of such treatment. Histone H1 shows virtually nothing at the elution position of ε-N-acetyllysine. However, there is a quite significant peak in this position in degraded HMG-E, as seen in the lower panel of Fig. 2. Again, the results for HMG-E were quite similar to those obtained for other HMG proteins subjected to such degradation. Further evidence that the protein digests contained free ε-N-acetyllysine was obtained as follows. A sample of degraded HMG protein was applied to the column of an amino acid analyzer. The effluent containing the presumptive ε-N-acetyllysine was diverted from the analyzer reaction coil and collected separately. The effluent was taken to dryness and hydrolyzed in 6 N HCl for 22 h. Subsequent amino acid analysis revealed the presence of lysine. Furthermore, the amount of lysine recovered was commensurate with the amount of ε-N-acetyllysine in the original effluent.

Radioactive Labeling of Calf Thymus HMG Proteins—Calf thymus HMG proteins, labeled with [3H]acetate as described under “Experimental Procedures,” were extracted and separated on sodium dodecyl sulfate-polyacrylamide gradient slab gels. The stained bands corresponding to HMG-1 and HMG-2 were cut out, combusted on a sample oxidizer, and counted for radioactivity. Fig. 3 shows the gel banding pattern of the HMG proteins as well as calf thymus histones that were run simultaneously and which served as markers. HMG-1 and HMG-2 were found to be significantly labeled and to virtually the same degree. The specific radioactivity of these proteins was approximately 4000 cpm/mg, and the total counts detected in HMG-1 and HMG-2 represented 40% of the counts found in a commensurate sample of total HMG proteins counted prior to separation of the components by gel electrophoresis.

**DISCUSSION**

The findings of the present investigation, provided by dinitrophenylation studies, extensive enzymatic digestion, and radioactive labeling experiments, are consistent with the view that HMG proteins, like some histones, exist in an acetylated form and that acetylation occurs as a postsynthetic modification of lysine residues. Dinitrophenylation of HMG proteins provided the first evidence that they might be acetylated or at least in some way modified at ε-amino functional groups. The results indicated that a relatively large percentage of ε-NH₂ groups in HMG proteins were unreactive toward fluorodinitrobenzene and one possible explanation for this would be that ε-NH₂ groups serve as sites for structural modification of the proteins. Conclusive proof of this premise was provided by trypsin and pronase digestion of the HMG proteins. In the course of these experiments, it was definitely determined that ε-N-acetyllysine is released from, and therefore is present in HMG proteins, just as it is present in histones (27, 28, 33). It follows, then, that the ε-amino groups of lysine must serve as acetylation sites. The results of in vitro labeling of calf thymus nuclei with [3H]acetate provided additional support for the conclusion that HMG proteins are acetylated. Furthermore, the significant acetate incorporation observed, even in the absence of appreciable HMG synthesis, showed that HMG proteins are subject to postsynthetic modification.

Coupled with their ability to bind DNA (15-17), the fact that HMG proteins apparently remain associated with the chromatin complex even at the nucleosomal level level of structure (13, 14) makes it tempting to implicate them in transcriptional control. The remarkable degree of sequence homology between HMG proteins from diverse sources (34, 35 and Footnote 2), as well as the estimation that there are $10^6$ to $10^7$ HMG molecules/nucleus (12) would seem to justify the contention that any role these proteins may play in transcription must involve a structural reorganization of the associated chromatin subunits.

It now seems possible that HMG proteins may be similar to histones in their binding to DNA and the manner in which this binding is regulated. Since postsynthetic acetylation occurs in HMG proteins as well as in histones, it is reasonable
to hypothesize that the same proposed mechanism of altering DNA-histone interactions through acetyl substitutions on the histone molecules (2–4) would also be in effect for DNA-HMG interactions. Acetylation may play a significant role in regulating a complex series of interactions between histones, HMG proteins, other non-histone nuclear proteins, and DNA, interactions which must change as the function of the chromatin in the cell nucleus changes.

The fact that acetylation is not confined to histones suggests that such protein modification may be a general mechanism of control of DNA binding by nuclear proteins. Experiments in progress are designed to test whether proteins as diverse in function as structural proteins, DNA unwinding proteins, polymerases, and possibly some nucleases may have in common a mechanism of regulating interactions with DNA by enzymatic alterations of their lysine residues.

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