On the phosphorylation of low molecular mass HMG (high mobility group) proteins in Ehrlich ascites cells

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This paper shows that the low molecular mass HMG proteins 14 and 17 do not seem to be phosphorylated in Ehrlich ascites cells whereas two other small HMG proteins designated HMG I and Y are. Amino acid analysis and peptide mapping of all four proteins demonstrated that HMG I and Y were not phosphorylated modifications of HMG 14 or 17.

HMG protein *Phosphorylation* *Proliferating cell*

1. INTRODUCTION

The low molecular mass HMG proteins 14 and 17 belong to a group of non-histone chromatine proteins [1] which, because of their possible role in transcription, have attracted a great deal of attention [2–7]. Their post-translational phosphorylation in proliferating cells have also been the subject of investigation by a number of groups [8–14]. Recent work from this laboratory has shown that in proliferating HeLa cells [12], HMG 14 and 17 were not phosphorylated, whereas two other phosphorylated low molecular mass HMG proteins designated HMG I and Y were present. On the other hand, several workers conclude that HMG 14 is phosphorylated in proliferating cells [8–11]. One group [13], which earlier reported that HMG 17 was phosphorylated in HeLa cells, has recently, in agreement with our own results and those of Walton and Gill [14], concluded that HMG 17 is not phosphorylated in HeLa cells.

Here, we have extended our investigation to cells from another species. Based on amino acid analysis and peptide mapping we have found that in Ehrlich ascites cells there exists, as in HeLa cells, a family of low molecular mass HMG proteins, two of which were phosphorylated.

2. MATERIALS AND METHODS

2.1. *Propagation of cells*

The Ehrlich ascites mouse cell line used was initially obtained from Professor H. Klenow, University of Copenhagen, and established as in vitro culture in this laboratory [15]. Cells were grown in suspension culture in Eagle's minimum essential medium, supplemented with 10% foetal calf serum, Hepes buffer (pH 7.3, final concentration, 15 mM), 1 ml 100 × non-essential amino acids to 100 ml, 100 units/ml penicillin and 100 μg/ml streptomycin. HeLa S3 cells were propagated in the same medium.

2.2. *Labelling of cells and isolation of nuclei*

Carrier-free [^{32}P]orthophosphate was added 3 h before harvesting to suspension cultures (0.03 mCi/ml) at a cell density 60×10^4/ml. Nuclei were isolated as in [16].

2.3. *Extraction of HMG proteins*

HMG proteins were either extracted from whole cells with 5% perchloric acid (final concentration) and precipitated with 6 vols acetone [17] or from cell nuclei with 0.35 M NaCl as in [17].

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2.4. *Polyacrylamide gel electrophoresis of proteins*

Acetic acid-urea gels containing 15% acrylamide and 0.4% bisacrylamide, 2.5 M urea and 0.9% acetic acid with a stacking gel containing 7.5% acrylamide, 0.2% bisacrylamide, 0.375 M potassium acetate buffer (pH 4.0) and 2.5 M urea were used [18]. Sodium dodecylsulfate (SDS)-polyacrylamide gels (15% acrylamide and 0.4% bisacrylamide) were run according to Laemmli [19]. All gels were stained with Coomassie brilliant blue G.

For preparative purposes, acetic acid-urea (as above) slab gel (15 cm wide and 1.5 mm thick) was used. Perchloric acid extracts from 5×10^8–10^9 cells were applied on the gel. Protein was eluted from gel strips as in [12].

To visualize ^{32}P-labelled proteins, Kodak X-omat RP films were exposed to dried gels, sometimes in combination with Siemens Titan 2HS intensifying screen at −70°C.

2.5. *Phosphatase treatment of ^{32}P-labelled proteins*

Protein from 2×10^7 cells was incubated at 37°C for 16 h with a mixture containing 100 mM Tris–HCl (pH 8.0), 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM PMSF and 10 μg *Escherichia coli* phosphatase (Sigma Type III R) in a total volume of 20 μl. Protein was precipitated with trichloroacetic acid and washed with acetone.

2.6. *Amino acid analysis*

About 3 μg protein were analysed at the central Amino Acid Analysis Laboratory, Institute of Biochemistry (Uppsala, Sweden).

2.7. *Digestion with* S. aureus *V8 protease and peptide mapping*

Perchloric acid soluble proteins were separated by acid-urea gel electrophoresis. The bands containing the low molecular mass HMG proteins were excised from the gel, stirred in water overnight, and finally for 1 h in the stacking gel buffer used in SDS-gel electrophoresis. The proteins in each equilibrated gel band were then subjected to proteolysis with *S. aureus* V8 protease during electrophoresis in an SDS gel [20]. Approx. 1 μg enzyme per well was used. The current was cut off twice for 30 min when the substrate and enzyme were stacked in the stacking gel to allow the digestion to occur.

3. RESULTS

In the following, the low molecular mass HMG proteins from [^{32}P]phosphate labelled Ehrlich ascites cells have been analyzed by acetic acid-urea gel electrophoresis and compared with those from HeLa cells and pig thymus. The electrophoretic pattern of the perchloric acid soluble proteins from Ehrlich ascites cells is shown in fig.1A (lane 1). It appears that the cells contain a protein in the same position as HMG 17 from HeLa cells (lane 2). In contrast to HeLa cells, Ehrlich ascites cells do not give a band in the position of HMG 14 from pig thymus (lane 3). There is, however, a distinct band slightly ahead of HMG 14 which in fig.1A is referred to as HMG 14_{EA}. Furthermore, it is seen that Ehrlich ascites cells contain two proteins with electrophoretic mobilities equal to those of HMG I and HMG Y in HeLa cells. The presence of HMG Y is seen more clearly in fig.1C. The autoradiograph of the acetic acid-urea gel is shown in fig.1B. There are two radioactive bands corresponding (lane 1′) to the positions of labelled HMG I and HMG Y from HeLa cells (lane 2′). However, no radioactivity was present in the positions corresponding to HMG 14 and HMG 17. Phosphatase treatment of the perchloric acid-soluble proteins from Ehrlich ascites cells removed all radioactivity from HMG I and Y as revealed by acetic acid-urea gel electrophoresis and autoradiography (not shown).

HMG I, Y, 14 and 17 in Ehrlich ascites cells were purified by preparative acetic acid-urea gel electrophoresis. The isolated proteins yielded only one band upon analysis in acetic acid-urea and SDS gels. Amino acid analyses of the proteins are recorded in table 1.

In order to compare quantitatively the amino acid compositions of HMG I, HMG Y, HMG 14_{EA} and HMG 17 from Ehrlich ascites cells and HMG 14 and 17 from calf thymus, the $S\Delta Q$ methods of Marchalonis and Weltman [21] were used. $S\Delta Q$ is a measure of the similarity in composition between two proteins, i.e., closely related proteins have small $S\Delta Q$ values. The different values are recorded in table 2. The low $S\Delta Q$ between HMG I and Y indicates great similarity in amino acid composi-

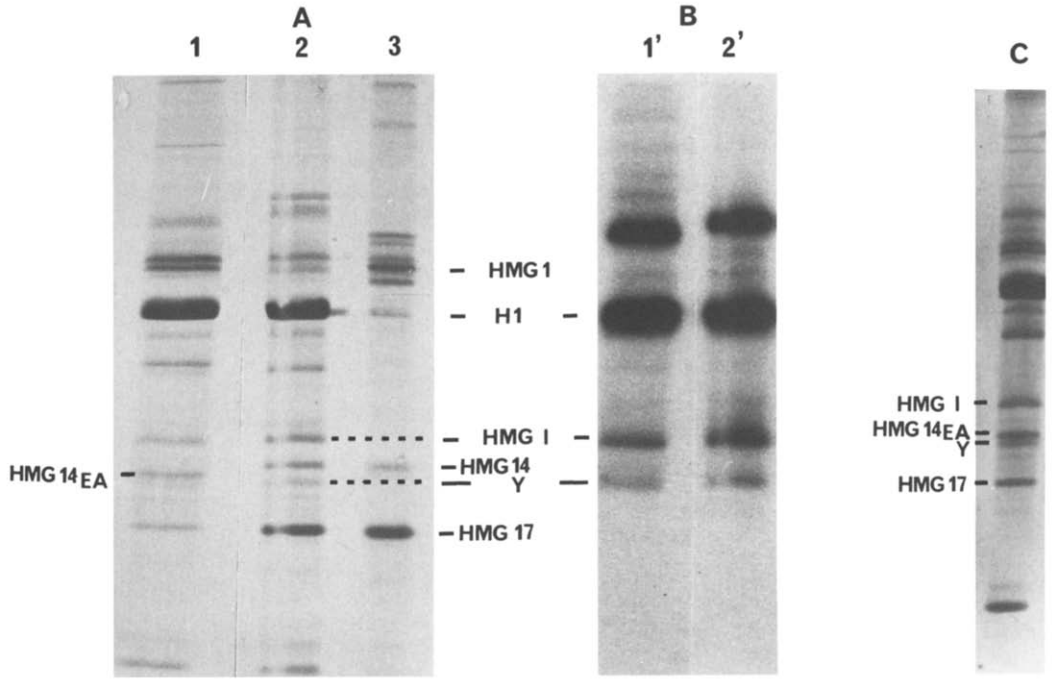

Fig.1. Comparison of perchloric acid extracted proteins from [^{32}P]phosphate labelled Ehrlich ascites and HeLa S3 cells by acetic acid-urea polyacrylamide gel electrophoresis. (A) Coomassie-stained proteins from interphase Ehrlich ascites cells (lane 1), interphase HeLa S3 cells (lane 2) and pig thymus (lane 3). (B) An auto radiograph of the gel in A. Lanes 1′ and 2′ correspond to lane 1 and 2, respectively. (C) Coomassie-stained proteins from Ehrlich ascites cells with a higher load of proteins.

tion. Furthermore, when comparing HMG I and Y with HMG 14_{EA} and HMG 17, respectively, the high $S\Delta Q$ values exclude the possibility that HMG I and Y are modified forms of HMG 14_{EA} and HMG 17. It is also seen that the amino acid compositions of, respectively, HMG 14_{EA} and HMG 17 from Ehrlich ascites cells are similar to those of HMG 14 and 17 from calf thymus (low $S\Delta Q$ values).

The proteins from Ehrlich ascites cells were characterized further by partial digestion with V8 protease. Fig.2 (lane f,g) shows that HMG I and HMG Y give very similar peptide patterns, each giving rise to 5 peptides. (The band just above band 1 probably represents undigested HMG Y and I, respectively.) When comparing HMG I and Y to HMG 14_{EA} and HMG 17 (lane e,c) there are striking differences in the peptide patterns. Furthermore, there is a close relationship between HMG 17 from calf thymus and Ehrlich ascites cells (lane b,c), each giving rise to two peptides with nearly the same electrophoretic mobility. On the other hand, HMG 14_{EA} and HMG 14 from calf thymus (lane e,d) give different digestion products.

HMG proteins should by definition be extractable with 0.35 M NaCl and soluble in 2% trichloroacetic acid [1]. To demonstrate that HMG I and HMG Y fulfilled these criteria, nuclei from Ehrlich ascites cells were extracted with 0.35 M NaCl. The soluble proteins were fractionated with 2% trichloroacetic acid and the resulting supernatant precipitated with 20% trichloroacetic acid.

Gel electrophoresis in acetic acid-urea of the precipitate revealed the presence of HMG I, HMG 14_{EA}, HMG Y and HMG 17 (not shown) confirming the view that they are HMG proteins.

4. DISCUSSION

Here we have shown that proliferating Ehrlich ascites cells contain a family of low molecular mass HMG proteins consisting of HMG 14, HMG 17

Table 1

The amino acid composition (mol%) of HMG proteins isolated from interphase Ehrlich ascites cells

	HMG I	HMG Y	HMG 14	HMG 17
Asx	2.9	3.6	8.7	11.8
Thr	5.6	5.3	3.6	3.7
Ser	11.9	10.8	9.0	5.0
Glx	18.1	17.8	14.3	10.0
Pro	11.2	9.9	7.0	12.1
Gly	11.6	11.3	9.3	11.6
Ala	6.0	6.6	16.1	16.9
Val	3.8	4.2	4.0	5.5
Met	–	–	–	–
Ile	1.2	1.6	1.5	–
Leu	3.3	2.9	3.3	2.1
Tyr	–	–	–	–
Phe	–	–	–	–
His	0.4	–	0.5	–
Lys	15.0	15.8	17.0	21.0
Arg	9.0	10.3	5.6	4.0
Asx + Glx	21.0	21.5	23.0	25.0
Lys + Arg	24.0	26.1	22.6	21.0

No corrections have been made for hydrolytic losses. The figures given represent the average of results from three different preparations

Table 2

$S\Delta Q$ values computed between the amino acid compositions of HMG proteins from Ehrlich ascites cells (table 1) and calf thymus [1]

	$S\Delta Q$
HMG I – HMG Y	7.1
HMG I– HMG 14_{EA}	201.0
HMG I – HMG 17_{EA}	382.5
HMG Y – HMG 14_{EA}	171.3
HMG Y – HMG 17_{EA}	346.8
HMG 17_{EA} – HMG 17 calf thymus	45.3
HMG 14_{EA} – HMG 14 calf thymus	43.4
HMG 14_{EA} – HMG 17 calf thymus	187.2

$$S\Delta Q = \sum_{i=1}^{N} (x_i - y_i)^2$$

in which x_i and y_i are the mol% of the ith amino acid in proteins x and y. N is the number of the different amino acids determined in the analysis

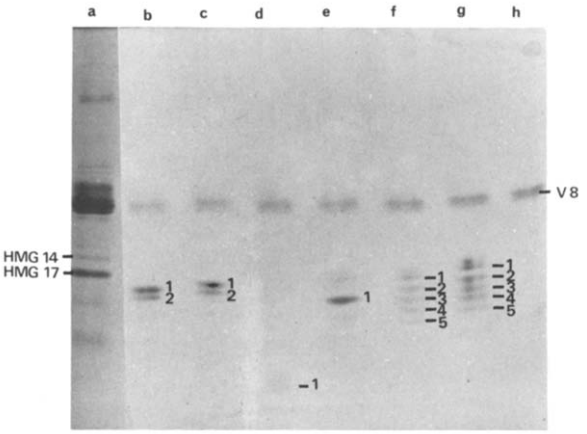

Fig.2. SDS gel electrophoresis of peptides obtained after digestion of HMG proteins with V8 protease. Perchloric acid-soluble proteins from calf thymus (lane a), peptides, from, respectively, calf thymus HMG 17, Ehrlich ascites HMG 17, calf thymus HMG 14, HMG 14_{EA} from Ehrlich ascites cells, HMG Y and HMG I (lanes b–g), V8 protease (lane h).

and two other phosphorylated proteins which are not modified forms of HMG 14 and 17. The amino acid compositions of the latter two proteins, HMG I and HMG Y, are very similar. Digestion with V8 protease gives similar peptide patterns in SDS gel electrophoresis suggesting that they are closely related. HMG I and HMG Y from Ehrlich ascites cells and HMG I and Y from HeLa cells [12] seem to be closely related since they have the same electrophoretic mobility, are phosphorylated and have similar amino acid compositions.

Our results show that HMG 17 from Ehrlich ascites cells and from calf thymus are very similar whereas HMG 14_{EA} and HMG 14 from calf thymus differ in electrophoretic mobility (fig.1) and peptides after digestion with V8 protease. This supports previous results [1] that HMG 14 is a protein with a less conserved sequence than HMG 17.

D'Anna et al. [22] have described an HMG protein from Chinese hamster ovary cells, which is phosphorylated and has an amino acid composition similar to that of HMG Y. Furthermore, Goodwin (personal communication) has found the presence of an HMG protein in rat fibroblast transformed cells with an amino acid composition similar to HMG I. These results together with our own on Ehrlich ascites and HeLa-cells suggest that

HMG I or HMG Y-like proteins occur in proliferating transformed cell lines from different species and that there exists a family of low molecular mass HMG proteins. Our finding that HMG 14 and 17 are not phosphorylated in proliferating Ehrlich ascites cells, is in agreement with previous results in HeLa cells [12], but in contrast to that reported by others [8,13,14]. We would like to suggest that phosphorylated proteins like hHMG $14_{\alpha}1$ and hHMG $14_{\alpha}2$ reported by Walton and Gill [14] and HMG 14b and HMG 14a described by Bhorjee et al. [13] for which no amino acid analyses are given, may correspond to HMG I and HMG Y and that phosphorylated HMG 14 and 17 from Ehrlich ascites cells reported by Saffer and Glazer [8] were mistaken for phosphorylated HMG I and Y.

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