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Mass Spectrometric Analysis of the HMGY Protein from Lewis Lung Carcinoma

IDENTIFICATION OF PHOSPHORYLATION SITES*

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The primary structure of the Lewis lung carcinoma protein HMGY belonging to the nuclear group of proteins HMGI (high mobility group I) was determined using electrospray and fast atom bombardment mass spectrometry. It was demonstrated that the sequence of the tumor protein corresponds to the amino acid sequence derived from the cDNA from cultured cells and that the N-terminal serine residue is *N*-acetylated. Moreover, the two high performance liquid chromatography-purified forms Y_1 and Y_2 of the protein HMGY were shown to differ at the level of serine phosphorylation, since they contain three phosphate and two phosphate groups, respectively, in the C-terminal region. No other modification was detected in the remaining part of the molecule.

A sub-group of low molecular mass, high mobility group (HMG)[1] nuclear proteins was first detected in HeLa S3 and Ehrlich ascites cells in 1983 (1). It is now known from several studies on normal and transformed cells, both murine and human, that this HMG sub-group contains three structurally related proteins named HMGI, HMGY (1-3), and HMGI-C (or HMGI') (4-7). Collectively they are called the HMGI proteins. The interest in HMGI proteins is based on the fact that they are expressed at high levels in proliferating cells and constitute a distinctive feature of undifferentiated or neoplastically transformed cells (1-8). Protein HMGI shows preferential binding to AT-rich DNA sequences (9-13), and presumably the other two related protein HMGY and HMGI-C have the same binding capabilities. Recently it has been shown that the HMGI protein binds to an AT-rich sequence upstream of the mouse lymphotoxin gene. Since this sequence acts as a transcriptional enhancing sequence, it has been suggested that HMGI is a transcription factor (14).

HMGI proteins are phosphoproteins (1, 2, 5, 6, 8, 15-22). The HMGI protein has phosphorylation sites for casein kinase at Ser-102 and Ser-103, both of which have been demonstrated *in vivo* and *in vitro* (2, 16, 18). This protein also exists in more extensively phosphorylated forms, since several additional modification sites have been observed at threonine residues 21, 53, 77, and 78 (16, 19, 22), two of which, Thr-53 and Thr-78 (present only in human), were shown to be p34/CDC2 kinase-dependent (19, 22). Moreover, it has been demonstrated that Ser-99 is a substrate of casein kinase II (18). The HMGY protein was identified as a single homogeneously phosphorylated protein (16, 17), which nevertheless can act as substrate for additional phosphorylation events (20).

In a previous paper we identified in Lewis lung carcinoma three proteins, which have been named HMGI-E, HMGI-D, and HMGI-C (23). The cDNA coding for HMGI-C, recently cloned and sequenced by us (7), demonstrates that this protein is structurally related to the HMGI/Y proteins (3); it has been suggested that the other two tumor proteins, HMGI-E and HMGI-D, probably correspond to the culture cell-derived proteins HMGY and HMGI, respectively.

This paper describes the structural characterization of the HMGI-E protein from Lewis lung carcinoma using advanced mass spectrometry procedures and demonstrates that the tumor protein is indeed identical to HMGY protein. Moreover, the two forms of HMGI-E, previously identified by HPLC (6, 23), are phosphorylated at serine residues in the C terminus and differ from each other by the presence of a single phosphate group. Both proteins lack the initial methionine and contain *N*-acetylserine at the N terminus. Hereafter, these proteins will be referred to as $HMGY_1$ and $HMGY_2$, which contain 3 and 2 phosphate groups, respectively.

MATERIALS AND METHODS

Protein Purification and Electrophoretic Analysis—HMG proteins were extracted from Lewis lung carcinoma together with histone H1 with 5% (g/100 ml) perchloric acid and acetone-precipitated (5, 8, 23). Reverse-phase HPLC protein fractionation was performed on a Bio-Rad RP-304 column using a Waters apparatus (6, 23). Polyacrylamide (15%) gel electrophoresis (250 × 0.7 mm) was carried out in acetic acid-urea essentially as described (5). Gels were stained with Coomassie Blue.

Enzyme Treatment—Trypsin (Sigma) and Lys-C (Boehringer) digestions of HPLC-purified proteins were carried out in 0.4% ammonium bicarbonate, pH 8.5, at 37 °C for 4 h. Glu-C (Boehringer) digestion was carried out in the same medium at 40 °C for 16 h. An enzyme/protein ratio of 1:50 (w/w) was used. Alkaline phosphatase treatment (3 units/mg protein) was performed in ammonium bicarbonate for 16 h.

Mass Spectra—FAB mass spectra were recorded with a VG ZAB 2SE double focusing mass spectrometer equipped with a cesium gun operating at 22 kV (2 μA). Samples (1-3 nmol) were dissolved in 0.1

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[1] The abbreviations used are: HMG, high mobility group; HPLC, high performance liquid chromatography; FAB/MS, fast atom bombardment mass spectrometry; ES/MS, electrospray mass spectrometry.

M HCl and loaded onto a glycerol-coated probe tip; thioglycerol was added just before insertion into the ion source. Spectra were recorded on UV-sensitive paper and manually counted. Assignments of the mass signals to the corresponding peptides along the cDNA sequence of protein HMGY were performed on the basis of their molecular mass. All the mass values recorded in FAB mode are shown as monoisotopic masses. Electrospray mass spectrometric analysis (24) of the intact HMGY proteins was performed on a VG BIO-Q triple quadrupole mass spectrometer. HPLC-purified samples (10 μl; 200/400 pmol) were injected into the ion source at a flow rate of 2 μl/min; the spectra were scanned from *m/z* 1000 to *m/z* 600 at 10 s/scan. Mass scale calibration was carried out by means of the multiple charged ions of a separate introduction of myoglobin; all data are shown as average masses.

RESULTS

Reverse-phase HPLC of perchloric acid-extracted protein from Lewis lung carcinoma separates the two sub-fractions, $HMGY_1$ and $HMGY_2$, of the protein HMGY. Alkaline-phosphate treatment of the extract results in the disappearance of the $HMGY_1$ and $HMGY_2$ peaks, and only one peak eluting at higher acetonitrile percentage can be observed (6). It has been suggested that $HMGY_1$ and $HMGY_2$ are two forms of the protein HMGY, which differ in the level of phosphorylation (6). Fig. 1 shows an acetic acid-urea gel electrophoresis of the total extract and Y_1 and Y_2 sub-fractions untreated (*lanes 1*, *3*, and *5*, respectively) and treated (*lanes 2*, *4*, and *6*) with alkaline phosphatase. As described previously (6), the phosphorylated proteins Y_1 and Y_2 migrate faster than the dephosphorylated proteins. This anomalous migration of phosphorylated $HMGY_1$ and $HMGY_2$ proteins in acid-urea gels could be due to a more compact conformation arising from intramolecular salt-bridges involving phosphate groups.

Lewis lung carcinoma-derived proteins $HMGY_1$ and $HMGY_2$ were analyzed using FAB/MS in order to both verify their amino acid sequences, which had been deduced from sequencing of the cDNA from cultured cells (3), and to determine the phosphorylation sites.

Three different enzymatic hydrolyses were performed on aliquots of HPLC-purified proteins using endoproteinase Lys-C, trypsin, and endoproteinase Glu-C. The resulting peptide mixture were directly analyzed by FAB/MS following the "FAB-mapping" procedure (25). The recorded mass signals were mapped onto the cDNA-derived HMGY sequence on the basis of their molecular mass and enzyme specificity. HMGY and HMGI differ by an insertion of 11 amino acid residues in the first half of the HMGI molecule (3). Since most of the previously described phosphorylation studies were carried out on the HMGI protein, we discuss sequence data according to the numbering of amino acid residues of this protein as obtained from cDNA sequencing (3). Fig. 2 shows cDNA sequences of the proteins HMGI and HMGY and summarizes the mass data that will be discussed later.

Mass spectral data for Lys-C and tryptic digests of $HMGY_1$ and $HMGY_2$ proteins are summarized in Table I; most of the mass signals could easily be assigned to the corresponding peptides along the protein sequence. However, the expected signal at *m/z* 725, corresponding to the N-terminal peptide 1–7 (see Fig. 2), was missing from the spectra. An apparently anomalous signal was detected at *m/z* 636 in both digests unmatched by any expected fragment according to the assumed cDNA sequence. This signal could be accounted for assuming that the initial methionine in HMGY proteins had been removed and the N-terminal serine residue had been *N*-acetylated. No signals mapping the C-terminal portion of the protein molecules could be detected in both the Lys-C and tryptic digests. This is probably due to the well known suppression phenomena always occurring in FAB/MS analysis of peptide mixtures (26, 27).

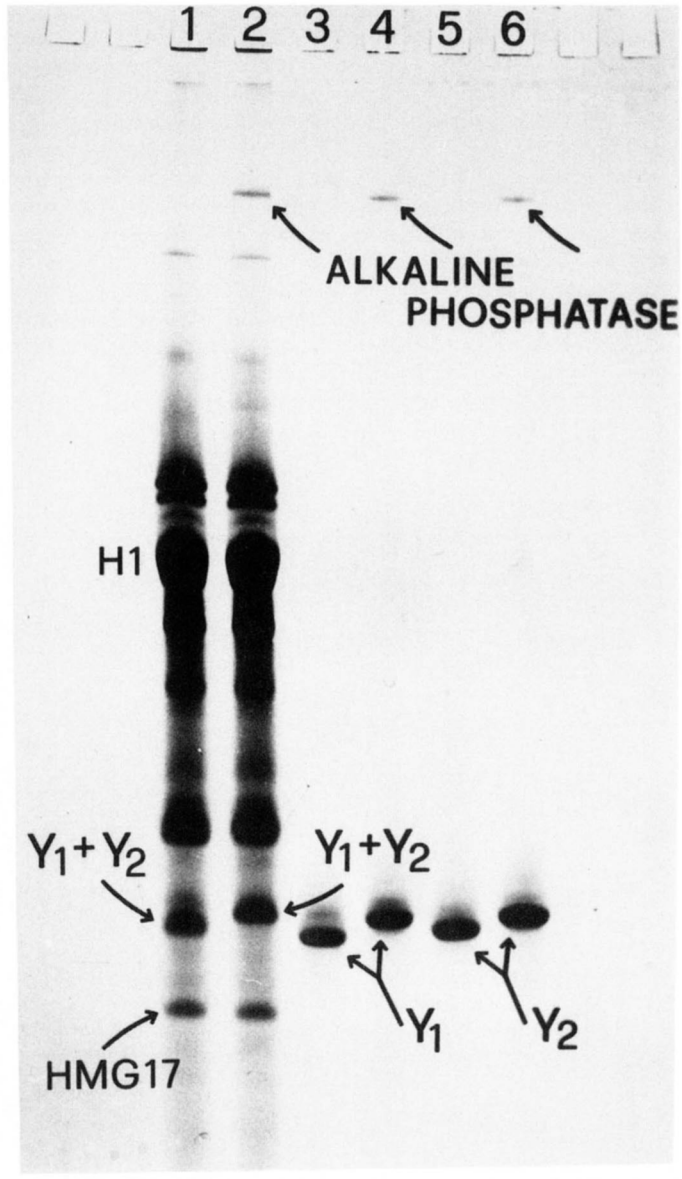

FIG. 1. **Acid/urea polyacrylamide gel electrophoresis of a protein extract from Lewis lung carcinoma and HPLC-purified fractions $HMGY_1$ and $HMGY_2$.** *Lanes 1* and *2*, total protein extract; *lanes 3* and *4*, $HMGY_1$; *lanes 5* and *6*, $HMGY_2$. *Lanes 1*, *3*, and *5*, untreated with alkaline phosphatase; *lanes 2*, *4*, and *6*, treated with alkaline phosphatase.

Table I also shows the FAB/MS data of the Glu-C digests of the proteins $HMGY_1$ and $HMGY_2$, and Fig. 3 shows the partial FAB spectra in the region where differences were observed. The signal at *m/z* 1382 of the $HMGY_2$ digest was tentatively assigned to the C-terminal G4 peptide 97–107 (see Fig. 2) originating from an incomplete protease cleavage and carrying two phosphate groups (expected mass value at *m/z* 1222; mass difference +160). This assignment was confirmed by incubation of the peptide mixture with alkaline phosphatase (28), which shifted the signal to *m/z* 1222 (data not shown).

According to the cDNA sequence (Fig. 2), fragment 97–107 contains 3 serine residues at positions 99, 102, and 103, respectively. Our data therefore indicate that 2 of these serines are modified by phosphate moieties *in vivo*. In fact, the spectrum of the Glu-C digest of the $HMGY_2$ also shows the presence of two more signals at *m/z* 533 (G5) and 662 (*G6*), both originating from the C-terminal region. These two signals correspond to the non-phosphorylated peptides 97–101

(G5) and 96–101 (G6), respectively, thus demonstrating that the serine residue at position 99 is unmodified. The mass spectrometric analysis, therefore, showed that the amino acid sequence of $HMGY_2$ protein does correspond to that predicted from cDNA sequencing and that this protein is phosphorylated at Ser-102 and Ser-103.

The FAB maps of $HMGY_1$ protein obtained by analyzing the Lys-C and tryptic peptide mixture were identical to those recorded for the $HMGY_2$ protein. However, when the Glu-C protease digest was mass-analyzed, several differences were detected (Fig. 3 and Table I). The mass signals at *m/z* 1382, 662, and 533 were absent in the spectra, whereas two new signals were detected at *m/z* 613 and 1462. These mass values were assigned to peptides 97–101 and 97–107, carrying one and three phosphate groups, respectively. Following alkaline phosphatase treatment, the signal at *m/z* 1462 shifted to *m/z* 1222 because of the removal of three phosphate moieties, thus confirming the above assignment. Since this fragment contains 3 serine residues, all of them must be phosphorylated in $HMGY_1$.

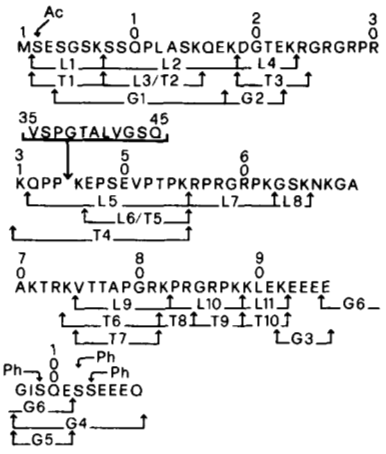

FIG. 2. **Amino acid sequences of murine HMGI/Y proteins derived from the cDNA sequences.** Alignment of the identified $HMGY_2$ fragments obtained with Glu-C (*G*), Lys-C (*L*), and trypsin (*T*) according to Table I is shown. Identified modifications: *Ac*, acetyl group (first serine of both $HMGY_1$ and $HMGY_2$); *Ph*, phosphate group (serines 102 and 103 of $HMGY_2$ and serines 99, 102, and 103 of $HMGY_1$).

The combination of FAB/MS data from different proteolytic digests allowed to verify about 80% of the entire sequence of the $HMGY_1$ and $HMGY_2$ proteins. Only a single possible phosphorylation site, Thr-72, was not mapped in the FAB spectra. However, this threonine residue does not seem to fulfill any known consensus sequence for protein kinases otherwise shown by serines 99, 102, and 103, which are embedded in phosphorylation motifs recognized by casein kinase II (29), and threonine 53, which can be phosphorylated by p34/CDC2 kinase (19, 22).

In order to confirm FAB/MS data and to verify the complete amino acid sequence of the two proteins, samples of $HMGY_1$ and $HMGY_2$ were submitted to electrospray mass spectrometric analysis. Both samples showed the characteristic bell-shaped distribution of multiply charged ions from which their molecular masses could be calculated. Fig. 4 shows the ES/MS spectra of $HMGY_1$ and $HMGY_2$ proteins following transformation of the multicharged ion spectra into real mass scale. The molecular mass of the intact $HMGY_1$ and $HMGY_2$ proteins were measured as 10765.74 ± 0.82 Da and 10686.48 ± 0.79 Da, respectively. The theoretical molecular masses obtained on the basis of the cDNA sequence and FAB/MS post-translational modification data were 10767.0 Da for $HMGY_1$ and 10687.4 Da for $HMGY_2$. The experimental molecular mass measurements are well in agreement with the expected mass value, thus confirming both cDNA sequencing data and post-translational modifications detected by FAB/MS. The measured mass values are consistent with the presence of the *N*-acetylserine at the N terminus of both proteins and the occurrence of 2 ($HMGY_2$) and 3 ($HMGY_1$) phosphorylated serine residues. Moreover, the ES/MS analysis ruled out the possible presence of any further modification of the protein structure.

TABLE I

Observed mass values of fragments obtained by digestion of Lewis lung carcinoma HPLC-purified proteins $HMGY_1$ and $HMGY_2$ with Lys-C (L), trypsin (T), and Glu-C (G)

Fragments carrying acetyl (Ac) or phosphate (Ph) groups are indicated. Alignment of the $HMGY_2$ fragments with the protein sequence deduced from the cDNA sequence is shown in Fig. 2.

MH^+	Glu-C fragment	MH^+	Lys-C fragment
Protein $HMGY_1$			
1433	G1: 4–17	636	L1: 2–7 (Ac)
549	G2: 18–22	1202	L2: 8–18
663	G3: 92–96	817	L3: 8–15
613	G4: 97–101 (Ph)	549	L4: 19–23
1462	G5: 97–107 (Ph)	1433	L5: 32–34/46–55
		983	L6: 47–55
		291	L7: 63–65
		829	L8: 75–82
		710	L9: 83–88

MH^+	Glu-C fragment	MH^+	Lys-C fragment	MH^+	Trypsin fragment
Protein $HMGY_2$					
1433	G1: 4–17	636	L1: 2–7 (Ac)	636	T1: 2–7 (Ac)
549	G2: 18–22	1202	L2: 8–18	817	T2: 8–15
663	G3: 92–96	817	L3: 8–15	705	T3: 19–24
1382	G4: 97–107 (Ph)	549	L4: 19–23	1561	T4: 31–34/46–55
533	G5: 97–101	1433	L5: 32–34/46–55	983	T5: 47–55
662	G6: 96–101	983	L6: 47–55	829	T6: 74–81
		866	L7: 56–62	701	T7: 75–81
		291	L8: 63–65	400	T8: 82–84
		829	L9: 75–82	457	T9: 85–88
		710	L10: 83–88	517	T10: 89–92
		517	L11: 89–92		

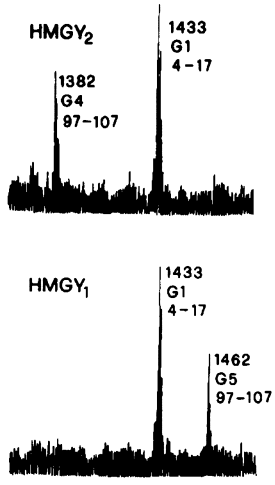

FIG. 3. **Comparison of FAB/MS spectra of the Glu-C digests of $HMGY_1$ and $HMGY_2$ proteins in the mass region where differences were observed.** Each mass signal correlates to the corresponding peptide along the protein sequence. *G*, Glu-C.

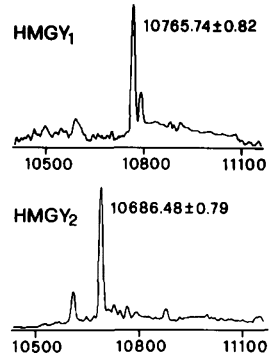

FIG. 4. **Transformed electrospray mass spectra of the $HMGY_1$ and $HMGY_2$ proteins.** The multiply charged ion spectra are transformed on a real mass scale. The molecular mass of the two proteins is reported as Da.

DISCUSSION

The amino acid sequence and post-translational modifications of the HMGI-E protein from Lewis lung carcinoma were determined using advanced mass spectrometric procedures. The primary structure of the tumor protein was shown to be identical to that deduced from cDNA sequence of culture cell-derived HMGY protein, thus demonstrating that HMGI-E and HMGY are indeed the same protein. Moreover, the mass spectral analysis showed that this protein does not have the initial methionine and contains *N*-acetylserine at the N terminus. This finding resembles that of histone H1 sub-types in which *N*-acetylserine is a rather common N-terminal residue (30–34).

Two forms of the HMGY protein, named $HMGY_1$ and $HMGY_2$, were identified in tumors and separated by HPLC (8, 23). Structural investigations by mass spectrometry showed that these two proteins have been modified in different ways by post-translational events at several serine residues in the C terminus. Both forms, in fact, contain phosphoserine residues at positions 102 and 103, whereas they differ in the phosphorylation of serine 99, which is unmodified in the Y_2 form. Phosphorylation of Ser-102 has already been observed in protein HMGI (2, 16, 18), and Ser-99 has been shown to be an *in vitro* substrate for protein kinase II (18). Therefore, tumor protein HMGY contains all the putative phosphorylation sites that are present in the HMGI protein sequence, and the $HMGY_1$ molecular species of this protein was indeed found in a hyper-phosphorylated form not previously described.

HMGY is a DNA-binding protein as the other proteins of the HMGI group (9–13). It is conceivable that the C-terminal domain is not involved in the binding because of the high content of negative charges, whereas this region might have a role in protein-protein interactions as an acidic transcriptional activator (35). In this respect, phosphorylation at the C-terminal domain adds four ($HMGY_2$) or six ($HMGY_1$) negative charges to the already existing nine from glutamic residues, thus strongly increasing the aptitude of these molecules for interaction with other positively charged proteins.

The accurate structural analysis of the proteins $HMGY_1$ and $HMGY_2$ showed that there were no further modifications in these proteins. In particular, threonine 53, which is known to be substrate of p34/CDC2 protein kinase, was found to be unmodified. Since HMGY protein was purified from a mixed population of tumor cells at different stage of the cell cycle, it is probable that only a small proportion is in the relevant stages of the cell cycle (S or G_2/M), and hence such phosphorylation would not be detected in our experiments.

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