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Identification of sites on chromosomal protein HMG-I phosphorylated by casein kinase II

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The amino acid sequence of a region on chromosomal protein HMG-I from human cells that is phosphorylated by casein kinase II has been determined. The sequence is: Leu-Glu-Lys-Glu-Glu-Glu-Glu-Gly-Ile-Ser-Gln-Glu-Ser(P)-Ser(P)-Glu-Glu-Glu-Gln. It corresponds to the C-terminal residues 90–107 of HMG-I [(1989) Mol. Cell. Biol. 9, 2114–2123]. Sequence analysis of the native peptide (90–107) after treatment, which specifically converts phosphoserine residues to *S*-ethylcysteine, revealed that 70–80% of serine residues 102 and 103 were phosphorylated in vivo. Both residues were fully phosphorylated in vitro by incubation with casein kinase II. These results suggest that casein kinase II is involved in the regulation of HMG-I function in the cells.

High-mobility-group protein; Casein kinase II; Protein phosphorylation; Phosphopeptide; Chromatin

1. INTRODUCTION

The high-mobility-group protein HMG-I is a nonhistone chromosomal phosphoprotein which is expressed at high levels in undifferentiated proliferating cells and which decreases in quantity when the cells differentiate terminally [1,2]. The phosphorylation level of this DNA-binding protein changes according to the stage of cell cycle being at its maximum at metaphase [3]. HMG-I and its isoform HMG-Y probably play an important role in cell division and may be involved in the condensation of A·T-rich regions of mammalian chromosomes and/or in maintaining the undifferentiated state of chromatin [2,4,5].

Casein kinase II (CK-II) is a ubiquitous, highly conserved, serine/threonine protein kinase found in the cytoplasm and nucleus of eukaryotic cells (see [6]). Increasing evidence suggests that CK-II plays a major role in signal transduction between extracellular mitogens and nuclear responses [6–9]. It is intriguing that a number of nuclear phosphoproteins implicated in cell proliferation, e.g. *myc, myb, fos*, E1A, and large T antigen, all possess potential CK-II phosphorylation sites and that *myc, myb* and large T antigen have been shown to be phosphorylated by CK-II in vitro [10,11].

We have previously shown that HMG-Y is phosphorylated in murine cells and that HMG-I(Y) from human and murine cells is not a preferred substrate for cyclic nucleotide dependent protein kinases or protein kinase C in vitro [12,13]. Since phosphorylation appears to be a major mechanism used by the cell to reversibly modify the activity of proteins, identification of kinases which phosphorylate HMG-I may contribute to our understanding of how HMG-I is regulated. In this study we have analyzed HMG-I purified from human cells as a potential substrate for nuclear casein kinase II.

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Abbreviations: CK-II, casein kinase II; HMG, high-mobility-group; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (3 Ci/mmol) was purchased from Amersham (England). Kemptide was obtained from Serva (Heidelberg, FRG) and a synthetic peptide substrate specific for CK-II from Peninsula Laboratories (Belmont, CA, USA). HPLC columns and reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, USA). The low-molecular-weight marker kit was purchased from Pharmacia (Uppsala, Sweden).

2.2. *Purification of HMG-I*

HMG-I was purified to homogeneity from 5% perchloric acid extracts of full-term human placentae using reversed-phase HPLC on a Bio-Rad RP-304 (C4) column with a water/acetonitrile gradient (7–20%) containing 0.1% (v/v) trifluoroacetic acid (TFA) [14]. The purity of HMG-I, which eluted at 14.8% acetonitrile, was checked by acetic acid/urea and SDS polyacrylamide slab gel electrophoresis [15,16].

2.3. *Purification of casein kinase II*

Casein kinase II (nuclear protein kinase II) was partially purified from isolated rat liver chromatin using a modification of the procedure of Inoue et al. [17]. The 0.4 M NaCl-extract of chromatin was

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processed as previously described by Palvimo et al. [12]. The specific activity of the enzyme assayed with the synthetic peptide substrate specific for CK-II (1.3 mM RRREEETEEE in the standard assay mixture) was 55 nmol of phosphate/min per mg at 30°C. CK-II did not phosphorylate Kemptide (LRRASLG).

2.4. *Phosphorylation of HMG-I and analysis of proteins*

The assay mixture contained 40 mM Tris-Cl, pH 7.5, 0.1 mM [γ-^{32}P]ATP (200–400 cpm/pmol), 9 mM $MgCl_2$, 140 mM NaCl, 2 mM 2-mercaptoethanol and 5% (v/v) glycerol, 17 μM HMG-I and 1.8 μg/ml CK-II. HMG-I was not dephosphorylated before phosphorylation assay. Reactions were initiated by adding radioactive ATP and terminated by adding concentrated sample buffer and analyzed by polyacrylamide gel electrophoresis. For quantification, the bands corresponding to HMG-I were cut out after brief staining-destaining, solubilized overnight and counted for radioactivity [13]. Phosphoamino acid analysis and tryptic peptide mapping of the phosphorylated protein were performed as previously described [13]. Protein was digested with thermolysin, fractionated by HPLC, and peptides were sequenced as in [2]. Phosphoserine residues were converted to *S*-ethylcysteine by β-elimination and addition of ethanethiol according to the procedure of Meyer et al. [18] as modified by Holmes [19].

3. RESULTS

HMG-I purified from human placentae was investigated as a potential substrate for CK-II. HMG-I was efficiently phosphorylated with a low concentration of the kinase. About one mol of [^{32}P]phosphate was incorporated per mol of HMG-I in 90 min (fig.1). The autoradiograms of acetic acid/urea- and SDS-polyacrylamide gels showed a single radioactive band comigrating with the Coomassie-stained protein band. Under the same conditions, ^{32}P-incorporation to HMG-14 was only about 1% of that to HMG-I (not shown). Serine was the only amino acid residue phosphorylated in HMG-I (fig.2A). Two-dimensional tryptic peptide map of the phosphorylated HMG-I revealed two major and two minor phosphopeptides (fig.2B). When the phosphorylated HMG-I was digested with thermolysin and the peptides were separated on a C18-column, two radioactive peaks eluting close to each other were observed (fig.3). Automatic Edman degradation of the major radioactive peak yielded the sequence shown in fig.4. The glutamic acid-rich phosphopeptide reported here is located at the C-terminal end of HMG-I (residues 90–107) [5].

The fact that the serine residues of the peptide eluted almost exclusively (95%) as phenylthiohydantoin-dithiothreitol-dehydroalanine (PTH-DTT-dehydroalanine) adducts at cycles 13 and 14 indicates the presence of phosphorylated serines in those positions. 97% of the phosphoserine standard (Sigma) eluted as a PTH-DTT-dehydroalanine adduct in our system, whereas a ratio of 43:57 (PTH-serine to PTH-DTT-dehydroalanine adduct) was found in the case of serine-5 of unmodified Kemptide-peptide and unphosphorylated serine residues of various peptides, as well as serine-99 of the major CK-II phosphorylated peptide (90–107). Interestingly, the other radioactive peak eluting immediately before the major peak, which contained 40% of total radioactivity, also gave the same sequence. This peak probably represents a peptide in which serine-99, in addition to serine-102 and serine-103, was phosphorylated. Thus, the heterogeneity detected in CK-II specific peptides of HMG-I in two-dimensional phosphopeptide analysis is probably due to the presence of peptide variants containing different combinations of phosphorylations at the three potential serine residues (fig.2B).

Since the labeled peptides contained three serine residues and, in our experiments, ^{32}P-incorporation was only about 1 mol phosphate per mol HMG-I, we investigated the presence of endogenous phosphates at

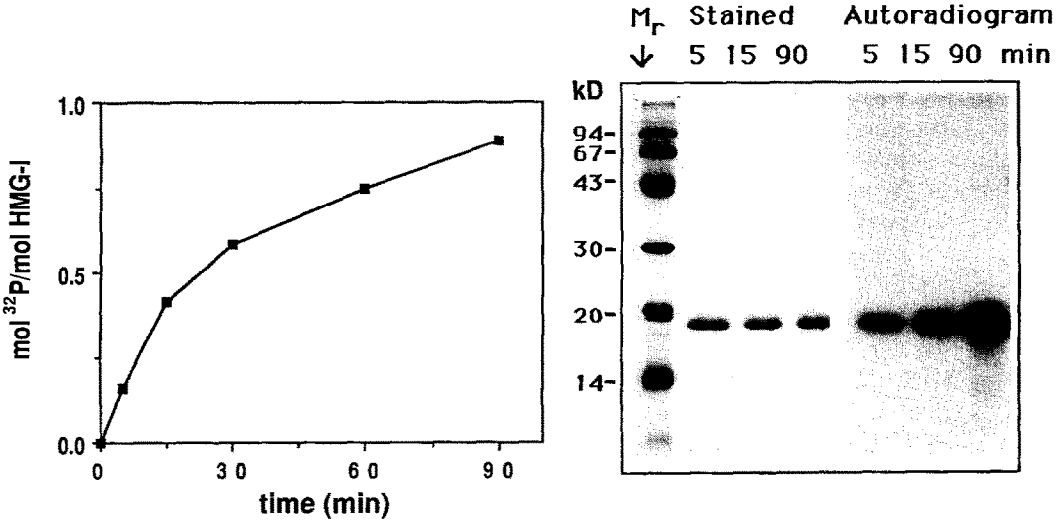

Fig.1. Phosphorylation of purified HMG-I from human placentae by casein kinase II from rat liver chromatin. The reactions were run as described in section 2. SDS-polyacrylamide (15%) gel electrophoretic analysis of the labeled proteins with low-molecular-weight marker proteins is shown on the right.

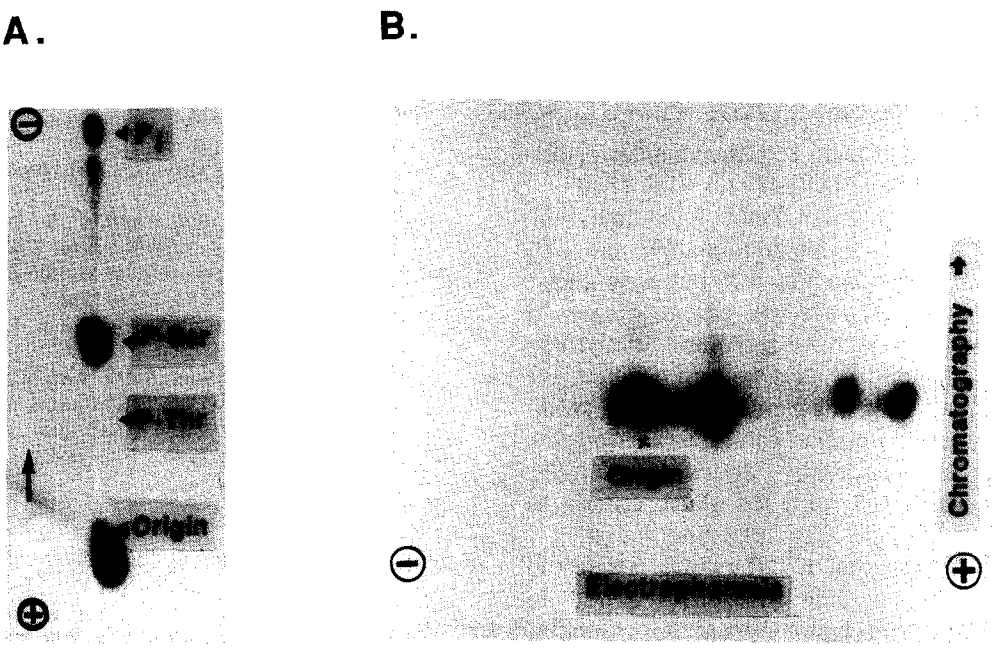

Fig.2. Phosphoamino acid analysis (A) and two-dimensional peptide map (B) of ^{32}P-labeled HMG-I. HMG-I was phosphorylated by CK-II for 80 min. The hydrolysate of phosphorylated protein (6 M HCl, 110°C, 2 h) was analyzed by thin-layer high-voltage electrophoresis (HVE) at pH 1.9 (2 kV, 45 min) and autoradiography (A). Tryptic phosphopeptides were separated on cellulose thin layer sheets using HVE at pH 3.5 (1 kV, 30 min) in the first dimension and ascending chromatography (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid, 30% H_2O) in the second dimension followed by autoradiography (B).

those positions. Without in vitro phosphorylation, 88% and 85% of the serine residues at cycles 13 and 14 eluted as their PTH-DTT-dehydroalanine adducts, whereas the respective value was only 57% at cycle 10. The presence of phosphoserines at positions 13 and 14 was confirmed by sequencing the same peptide after modification with ethanethiol, which specifically modifies phosphoserine to S-ethylcysteine. PTH-*S*-ethylcysteine, eluting just before diphenylthiourea [18], was present at cycles 13 and 14, but not at cycle 10. We interpret the data to indicate that HMG-I from human placenta contained without in vitro phosphorylation about 0.7 mol phosphate at serine-102 and 0.8 mol phosphate at serine-103 per mol of the CK-II-preferred peptide. After an 80 min incubation with CK-II, both serines were fully phosphorylated to 1 mol/mol and serine-99 to 0.3 mol/mol.

4. DISCUSSION

The sequence of human HMG-I cDNA has been recently reported [5]. The cDNA encodes a protein containing 107 amino acids whose sequence is virtually identical to the amino acid sequence of human placental HMG-I determined by peptide fragment sequencing

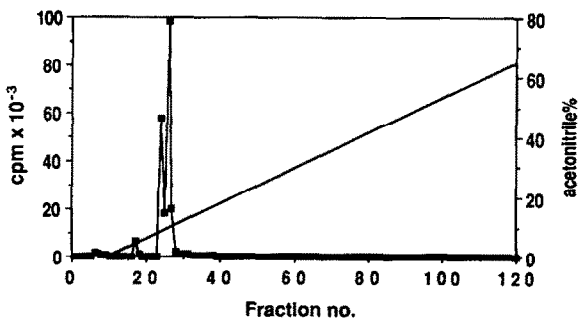

Fig.3. Separation of ^{32}P-labeled peptides generated by digestion of HMG-I with thermolysin on a Bio-Rad RP-318 column using a 0–70% linear acetonitrile gradient containing 0.1% TFA in 60 min. The fractions (30 s/0.5 ml) were counted for radioactivity as Cerenkov radiation.

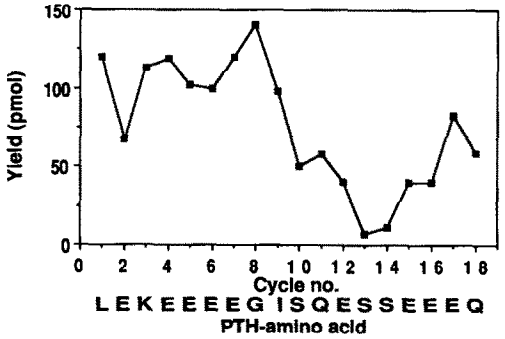

Fig.4. Sequence analysis of the major thermolytic phosphopeptide of HMG-I phosphorylated by CK-II. Yields of amino acids identified in cycles are plotted vs cycle number. The peptide was sequenced using an Applied Biosystems 477A sequencer connected to 120A (on-line) PTH-amino acid analyzer.

[20]. The protein designated as HMG-Y has an amino acid sequence identical to that of HMG-I except for an internal deletion of 11-amino acids [1,5]. HMG-I(Y) structure appears to be well-conserved during evolution; the deduced amino acid sequence similarity between murine and human HMG-I(Y) is 97% [1,5].

According to our results, HMG-I contains endogenous phosphates at serine residues 102 and 103, which are also phosphorylated in vitro by casein kinase II. The consensus sequence of casein kinase II substrates consists of a serine or threonine followed by a cluster of glutamic and/or aspartic acid residues, the residue at position +3 playing a crucial role [21]. Additionally, phosphoserine residues can replace carboxylic acid residues as specificity determinants [22]. Serine-102 and serine-103 fulfil the above criteria as well as serine-99, if serine-102 is phosphorylated.

Current data suggest that CK-II, which is released from chromatin at an ionic strength required to dissociate HMG proteins from chromatin, phosphorylates HMG-I and probably also HMG-Y in vivo and is thus involved in the regulation of function of these proteins. The addition of a negative charge to HMG-I(Y) (the net charge of this region, i.e. residues 90–107, is −8 without phosphorylation and −14, if fully phosphorylated) may strengthen the interaction between the highly acidic C-terminal tail of HMG-I(Y) and the basic histones and take part in condensation/repression of chromosomal structures during mitosis. We have previously shown that phosphorylation of HMG-14, which is also a basic protein with a highly asymmetrical charge distribution, at its acidic C-terminal region increases considerably its binding affinity for histone H1 [23]. The CK-II-mediated phosphorylation of HMG-I may represent one of the targets of the circuitry involved in mitogenic signal transduction and may act in concert with some phosphorylated nuclear proto-oncogene products like c-*myc* which is also present at high levels in undifferentiated cells and which decreases in quantity when the cells differentiate terminally [10,11,24].

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