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THE METAPHASE SPECIFIC PHOSPHORYLATION OF HMG I

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SUMMARY: In vivo labelling of HeLa cells arrested in metaphase with [^{32}P]-phosphate and in vitro phosphorylation of HMG I with the partially purified growth assosciated H1 kinase was used to study metaphase specific phosphorylation of HMG I. It was found that threonine 53 and 78 became phosphorylated. These amino acids are embedded in respectively the sequence PTPKR and TPGRK which are similar to the sequences phosphorylated by the growth associated H1 kinase.

The high mobility group chromosomal protein HMG I (N-terminally blocked) has been characterized by amino acid sequence determination (1,2) and is phosphorylated at several sites at mitosis (3). The mitotic phosphorylated protein is designated HMG I_m. Cloning of c-DNA for HMG I has recently been reported (4,5). HMG I binds preferentially to A/T-rich double stranded DNA sequences in vitro(6,7). High affinity binding sites have been detected in the α-satellite 172 bp repeat in African Green Monkey cells and in 3'-regions of several genes (6,8,9). Furthermore it has been reported that a HMG I like protein binds to rat rDNA enhancer and stimulates RNA polymerase I transcription (10). It also binds to cis acting sequences from mouse rDNA (11). In human and mouse metaphase chromosomes HMG I seems to be localized to G(Q) and C-bands and may be involved in the condensation of A/T-rich regions in the centromere/or telomer of mammalian chromosomes(12).

A growth associated kinase which phosphorylates H1 at sites which are phosphorylated at mitosis in vivo, has been partially purified (13). The sites which are phosphorylated are embedded in sequences S/TPK or S/TPXK which are similar to the

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Abbreviations used: HPLC, high performance liquid chromatography,
PTH-amino acids, phenyl thio hydantoin derivatives of amino acids.

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sequences TPKR and TPGR found in HMG I. We have therefore isolated the growth assosciated H1 kinase according to the method of Langan and used it to phosphorylated HMG I in vitro. The sites phosphorylated by this kinase have been compared to the sites phosphorylated in HMG I at mitosis.

MATERIALS AND METHODS

Propagation of cells and labelling of cells with [^{32}P]-phosphate: HeLa S3 cells were propagated at a density to 50x10^4/ml and arrested in metaphase with colcemid as in (3). Carrier free [^{32}P]-phosphate was added 3h before harvesting (0.03mCi/ml).
Extraction and purification of HMG I: Proteins were extracted from HeLa cells with 5% perchloric acid and purified by phosphocellulose chromatography and HPLC as in (1).
Enzymatic cleavage:40 µg protein was digested with respectively thermolysin and trypsin (1 µg of each) in 20 µl of 0.1 M NH_4HCO_3 at 37°C for 2h. The peptides obtained were purified on HPLC (2).
Sequence determination and determination of phosphorylated amino acids:Peptides were sequenced by automated Edman degradation using a pulsed phase sequencer, Applied Biosystem 477 A ,as described in (1). A portion of the labelled peptide was degraded in a spinning cup sequencer and the product obtained at each cycle was counted for [^{32}P]-phoshate without conversion to the PTH-derivative, as described (14).
Phosphoserine/phosphothreonine determination:Samples were treated with 6N HCl for 2h at 110° C in vacuum, and then run on silica plates at pH 1.9 for 1h at 1000 V.
Polyacrylamide gel electrophoresis: Acetic acid urea gel electrophoresis was performed with a stacking gel containing 7.5% acrylamide and a resolving gel containing 15% as in (2).
Purification of the growth associated H1 kinase: Chromosomes were obtained from HeLa S3 cells arrested in metaphase as described by Paulson and Taylor (15).Kinase activity was purified from the chromosomes as described by Langan (13), with 0.4 N NaCl extraction and fractionation with ammonium sulphate precipitation.The fraction containing the H1 kinase was finally fractionated by calcium phosphate gel and the fraction between o.1M and o.2M phosphate was collected.
Phosphorylation in vitro with purified H1 kinase: The reactions were carried out for 15 min at 30°C in a buffer containing 10 mM $MgCl_2$, 20 mM Hepes pH 7.5, 1 mM dithiothreitol, and [^{32}P]-ATP was added to a final concentration of 50 µM and 2 µCi per reaction.

RESULTS AND DISCUSSION

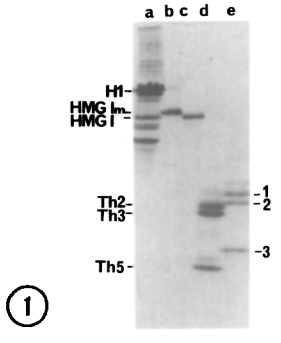
HMG I_m was purified from [^{32}P]-labelled He La S3 cells arrested in metaphase.Perchloric acid extracted proteins were separated on phosphocellulose and HMG I_m was finally purified on HPLC (Fig.1 lane b).Purified HMG I_m was then digested with thermolysine and the fragments compared with the thermolytic fragments obtained from HMG I by acetic acid urea gel electrophoresis (Fig.1 lane d). Fragments Th 2, Th 3 and Th 5 obtained from HMG I have been sequenced in previous work (1) and were shown to contain the

following sequences:LASKQEKDGTEKRGXGRPRKQPPVSPGTA (Th 2, amino acids 12-40), LVGSQKEPSEVPTPKRPRGRPKGSKNKG (Th 3, amino cids 41-68) and TTTPGRKPRGRPKK (Th 5,amino acids 76-89). The numbers given refer to the amino acids derived from cDNA for HMG I (4). The three peptides 1, 2 and 3 obtained from HMG I_m had lower mobility (Fig. 1, lane e) than the peptides from HMG I (Fig. 1, lane d), an expected difference due to mitotic phosphorylation. It is seen from the autoradiograph (Fig. 2, lane c) that the three peptides were labelled.

Peptide 2 was purified by HPLC (Fig.2 lane d). Amino acid sequencing confirmed that it was identical to Th 3. Partial acid hydrolysis of peptide 2 and thin layer electrophoresis demonstrated the presence of only phosphothreonine (Fig.3).Since Th 3 and hence peptide 2 contain only one threonine, Thr 53 is phosphorylated in HMG I at mitosis.

Peptide 3 (Fig. 2, lane e) eluted from HPLC at about the same position as Th 5, and had the same mobility in acetic acid urea gel electrophoresis (Fig. 5, lane b) as Th 5 phosphorylated in vitro with purified kinase. Peptide 3 is therefore most likely phosphorylated Th 5.

The growth associated H1 kinase was used to phosphorylate HMG I in vitro. The kinase fraction is reported to be free of c-AMP



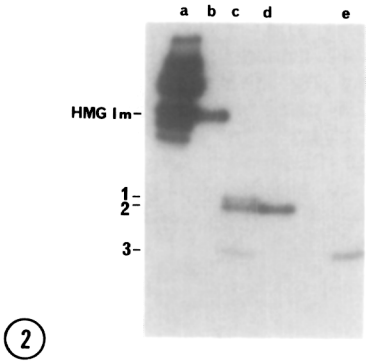


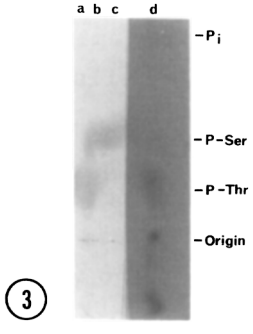
Fig.1.Acetic acid urea gel electrophoresis (coomassie stained) of thermolytic fragments derived from HMG I and HMG I_m.Lane a,perchloric acid soluble proteins from human placenta. Lane b, HMG I_m purified from HeLa cells arrested in metaphase. Lane c, HMG I purified from human placenta. Lane d and e, thermolytic fragments from HMG I and HMG I_m respectively.

Fig. 2.Acetic acid urea gel electrophoresis (autoradiograph) of thermolytic fragments of HMG I_m. Lane a, perchloric acid soluble proteins from HeLa cells arrested in metaphase. Lane b, purified HMG I_m. Lane c, thermolytic fragments from HMG I_m. Lane d and e, peptides 2 and 3 purified by HPLC.

dependent kinase (13) and did not contain casein kinase II as the phosphorylation was not inactivated by heparin (not shown). HMG I was phosphorylated by the kinase, then digested with trypsin and the tryptic fragments were purified by HPLC. A labelled fragment eluting in the position of peptide Tr 1 (with the sequence KTTTTPGR, amino acids 74-81) described previously (1) and which overlaps with Th 5 (amino acids 76-89), was subjected to automated Edman degradation. The radioactivity released at each cycle was counted and Thr 78 was identified as the phosphorylated amino acid (Fig. 4). Peptides Th 2, Th 3 and Th 5 from placenta HMG I were also used as substrates for the kinase, and it was found that only Th 3 and Th 5 were phosphorylated (Fig. 5, lanes d and e).

In the present work we have shown that amino acid Thr 53 is one of the amino acids which is phosphorylated at mitosis. Thr 53 is embedded in the sequence TPKR which is similar to sequences phosphorylated by the growth associated H1 kinase (13,16). Another site, Thr 78 in HMG I is phosphorylated by the growth associated H1 kinase. This site might be phosphorylated in metaphase since peptide Th 5 which contains Thr 78 (amino acids 75-89) was found to be phosphorylated in vivo.The sequence surrounding Thr 78 is also similar to sequences phosphorylated by the growth associated H1 kinase.

It is interesting that Thr 53 and 78 are close to sites which have been suggested as A/T- binding sites in HMG I (1). It should



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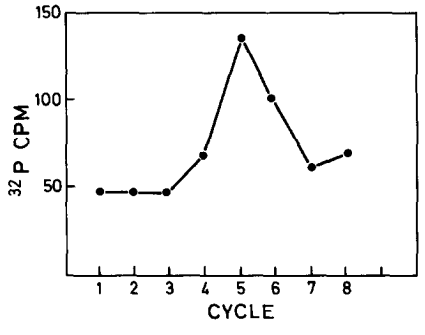

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Fig.3.Phosphoamino acid analysis of peptide 2 derived from [^{32}P]-labelled HMG I_m. Hydrolysate was analysed by thin layer high voltage electrophoresis.Lane a, unlabelled phosphothreonine. Lane b, a mixture of phosphothreonine and phosphoserine and the hydrolysate of peptide 2. Lane c, unlabelled phosphoserine.Lane d, autoradiograph of lane b.

Fig.4.Determination of phosphoamino acids in the tryptic fragment Tr 1 derived from HMG I phosphorylated by the growth associated H1 kinase.Automated Edman degradation of the peptide was performed and the radioactivity released in each cycle was counted.

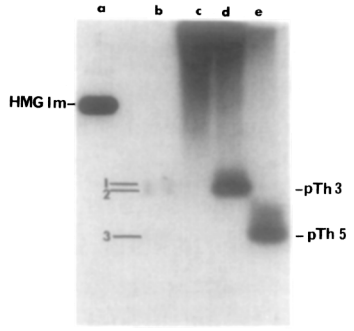

Fig.5.Thermolytic fragments of HMG I phosphorylated by the growth associated H1 kinase analyzed by acetic acid urea gel electrophoresis.The autoradiograph is shown.Lane a, HMG I_m from HeLa cells arrested in metaphase, Lane b, thermolytic fragments derived from labelled HeLa HMG I_m. Peptide Th 2(lane c), peptide Th 3 (lane d) and peptide Th 5(lane e) were phosphorylated by the kinase.

also be noted that the sequence SPK(R)K(R) is suggested to be responsible for the binding of proteins to the minor groove of double stranded A/T-rich regions (17). This sequence is almost identical to the sequence TPKR in HMG I which contains phosphorylated Thr 53. The mitotic phosphorylation of this amino acid may therefore cause dissociation between part of HMG I and A/T rich region in DNA, thereby exposing sites which are free to interact with other molecules.

When the thermolytic fragments from HMG I_m were fractionated on HPLC,one peptide in addition to peptides 1,2 and 3 were labelled. This peptide eluted in HPLC equal to Th 6 (1) and did not because of its acidity exhibit mobility in the acetic acid urea system. Th 6 is phosphorylated by casein kinase II in vitro at amino acids 102 and 103 (18) and is phosphorylated in interphase cells (1) and thus may be phosphorylated during the cell cycle.

It has been suggested (19) that the growth associated H1 kinase is similar to the $p34^{cdc2}$ kinase. The amino acids phosphorylated by the $p34^{cdc2}$ kinase seems to be embedded in the sequence: polar/basic-Thr/Ser-Pro-X-basic (20). Amino acid Thr 78 in HMG I is embedded in a sequence which is identical to this sequence, while the sequence around Thr 53 is identical except for the N-terminal prolin. It would therefore be of interest to investigate if HMG I could be a substrate for a highly purified $p34^{cdc2}$ kinase.

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