PHOSPHORYLATION OF HMG 17 BY PROTEIN KINASE NII FROM RAT LIVER CELL NUCLEI

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1. Introduction

Chromatin contains a group of non-histone proteins known as the high mobility group (HMG) proteins. They consist of four main proteins: HMG 1, HMG 2, HMG 14 and HMG 17 [1].

Since limited DNase 1 digestion of chromatin causes a selective fragmentation of the active chromatins with a concomitant and preferential release of HMG proteins, it has been postulated that the actively transcribing chromatin regions are enriched in the HMG proteins relative to the inactive chromatins [2,3]. In fact, HMG 14 and HMG 17 were shown to confer a DNase I-sensitive structure which is characteristic of the active chromatin [4,5]. Similarly, micrococcal nuclease digestion rapidly releases HMG 1 and HMG 2 from the active chromatin, suggesting that these proteins are located in the nucleosomal linker regions of the active chromatin [3,6,7].

Changes in chromatin activity for RNA synthesis are strongly correlated with the alteration in the phosphorylation of nuclear chromosomal proteins (reviewed [8]). Therefore, it might be interesting to investigate the possibility of whether HMG proteins undergo a phosphorylation–dephosphorylation reaction during the changes in gene expression.

In rat liver cell nucleus, there are two protein kinases which are not regulated by cyclic nucleotides. They have been designated NI and NII [9], and recently purified to homogeneity [10,11]. The physiological substrates for each enzymes are, however, still unknown.

We report here on the phosphorylation of isolated HMG proteins by partially purified nuclear protein kinase NII. In contrast to NI kinase, NII enzyme was found to phosphorylate HMG proteins to a high degree. The protein species phosphorylated by NII kinase was HMG 17 together with a protein which was probably HMG 14. Neither HMG 1 nor HMG 2 served as substrates for protein kinase NII.

Abbreviation: PMSF, phenylmethylsulfonyl fluoride

2. Materials and methods

2.1. *Isolation of HMG proteins*

Finely minced rat liver tissues (43 g) were homogenized in 2 vol. 2.3 M sucrose, 3 mM $MgCl_2$, 0.2 mM PMSF using a Potter-type homogenizer and mixed with 7 vol. of the same sucrose solution. After the homogenate was centrifuged at 40 000 × *g* for 60 min, the nuclear pellet was washed with 100 ml portions of 0.3 M sucrose, 3 mM $MgCl_2$, 0.2 mM PMSF, 12 mM Tris–HCl (pH 7.6) once, and twice with 75 mM NaCl, 0.25 mM PMSF. The washed chromatin was then suspended in 50 ml 0.35 M NaCl, 0.25 mM PMSF, 5 mM Na-phosphate (pH 6.75) and homogenized using a loose-fitting Potter type homogenizer with 40 strokes by hand. The homogenate was centrifuged, and the supernatant was adjusted to 5% perchloric acid by adding a 60% solution. After centrifugation, 7 vol. cold acetone was added to the supernatant, and the mixture was stirred slowly for 1 h at 0°C and then kept at −20°C for 15 h. The precipitates were collected by centrifugation, washed with acetone and dried.

2.2. *Extraction and partial purification of nuclear protein kinases NI and NII*

Rat liver nuclei were isolated from 210 g tissue as above, and successively washed in 50 mM NaCl, 10% (v/v) glycerol, 15 mM mercaptoethanol, 20 mM Tris–HCl (pH 7.2). The nuclear protein kinases were

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extracted from the chromatin with 150 ml 0.4 M NaCl, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 20 mM Tris–HCl (pH 7.85) in a similar manner as HMG protein extraction, and dialyzed against 30 mM $(NH_4)_2SO_4$ in buffer A (25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM mercaptoethanol, 0.2 mM PMSF, 50 mM Tris–HCl (pH 8.0)). After removing the precipitates by centrifugation, the supernatant was applied to a DEAE–Sephadex column (2.66 × 38.0 cm). NI kinase was recovered at the flow-through fractions; NII kinase was eluted at 175 mM $(NH_4)_2SO_4$ with a linear salt concentration gradient. The NI kinase was successively applied to a phosphocellulose column (1.6 × 13.7 cm) which was pre-equilibrated with 40 mM $(NH_4)_2SO_4$ in buffer B (buffer A minus 5 mM $MgCl_2$). The kinase was eluted at 125 mM $(NH_4)_2SO_4$ with a linear concentration gradient in buffer B. The protein kinases from the ion-exchange columns were precipitated with $(NH_4)_2SO_4$ at 63% saturation, dissolved in 10 ml of 0.4 M NaCl in buffer A″ (same as buffer A except 10% (v/v) glycerol), and further purified on a Bio-Gel A-1.5m column (2.12 × 126 cm, Bio-Rad). The enzymes recovered from the column were precipitated with $(NH_4)_2SO_4$ at 75% saturation, dissolved in 3 ml buffer A and used as the nuclear protein kinase preparations. After the chromatography, NI and NII enzymes were both purified 200–300-fold.

2.3. *Preparation of non-histone proteins*

The flow-through fraction of phosphocellulose column chromatography above was adjusted to 65% $(NH_4)_2SO_4$ saturation. The precipitated proteins were collected by centrifugation, dissolved in 11.5 ml 0.4 M NaCl in buffer A″, and dialyzed against the same solution. The content recovered after centrifugation was used as a non-histone protein preparation (2.3 mg/ml).

3. Results

We employed here an in vitro phosphorylation system with isolated HMG proteins as substrate and partially purified nuclear protein kinases as enzyme to:

(i) Examine whether the HMG proteins could undergo phosphorylation;

(ii) If so, to determine which nuclear protein kinase is responsible for the reaction;

(iii) To avoid serious degradation of the HMG proteins which actually happened when the isolated nuclei were incubated with [γ-^{32}P]ATP.

Fig.1 shows phosphorylation of HMG proteins by partially purified rat liver nuclear protein kinases. As a reference substrate, a whole histone preparation was employed. It is apparent from the figure that, while NI kinase only marginally phosphorylated the substrate proteins, NII kinase remarkably phosphorylated both the HMG proteins and the histone preparation.

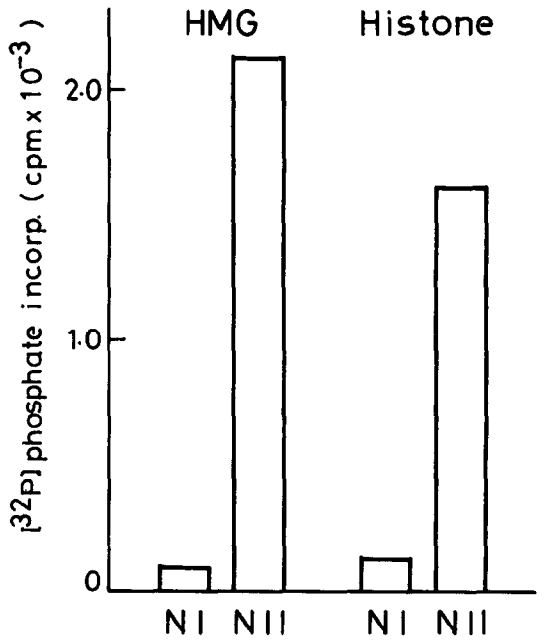

Fig.1. Phosphorylation of rat liver HMG protein and whole histone preparations by partially purified protein kinase NI and NII. 5 μl protein kinase (NI or NII), 13.2 μg substrate proteins (HMG proteins or whole histones) and 10 μM [γ-^{32}P]ATP (0.05 μCi) were incubated at 30°C for 10 min in a total 100 μl 12.5 mM $MgCl_2$, 40 mM Tris–HCl (pH 7.2). After the incubation, the mixture was chilled to 0°C, 50 μg carrier histones (50 μl) and 3 ml 30% trichloroacetic acid was added. The mixture was vortexed, and the precipitates formed were collected under suction on a glass filter (Whatmann, 2.4 cm GF/F) which was prewashed with 20% trichloroacetic acid. The glass filters were subsequently washed extensively with 20% trichloroacetic acid followed by 5 ml methanol, dried and counted in a toluene scintillator solution. Under the assay conditions with casein employed as a standard substrate at 1 mg/ml, NI kinase transferred 11.0 pmol [^{32}P]ATP phosphate (2.22 × 10^4 cpm), while NII kinase transferred 9.1 pmol (1.81 × 10^4 cpm), indicating that the NI enzyme activity was slightly higher than that of NII kinase (with a casein substrate).

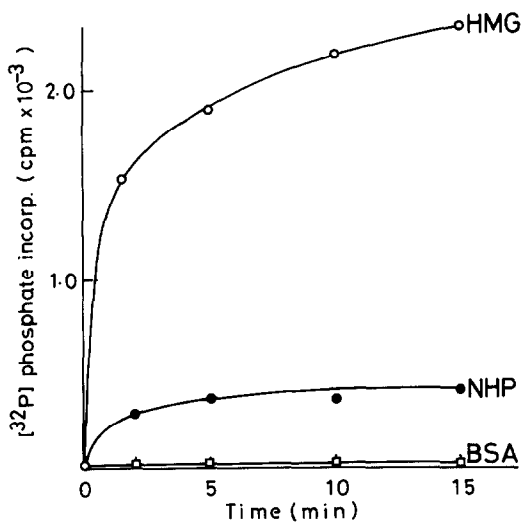

Fig.2. Phosphorylation of protein kinase NII of rat liver HMG proteins, non-histone proteins and bovine serum albumin. The phosphorylation reaction was done similarly to that in fig.1 except that the incubation time was varied. Substrates were rat liver HMG proteins (○), rat liver non-histone proteins (●) and bovine serum albumin (□).

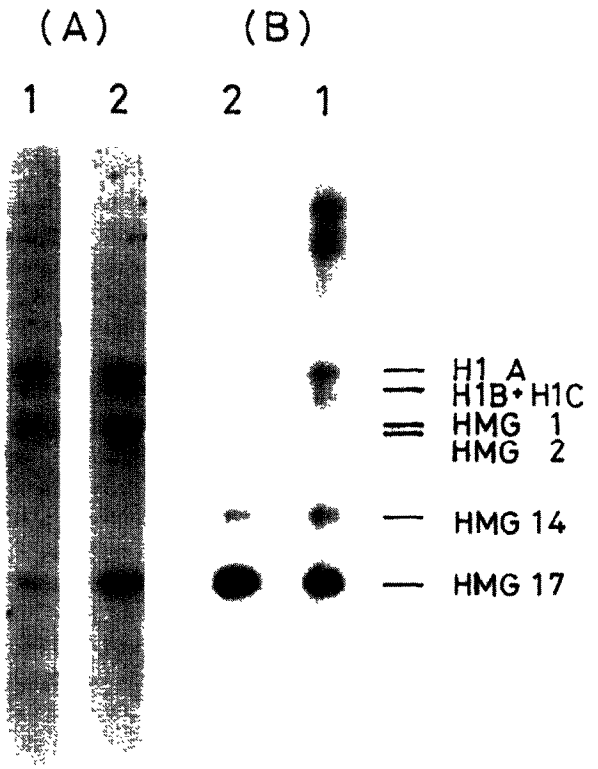

Fig.3. SDS–polyacrylamide gel electrophoresis and autoradiography of ^{32}P-labelled HMG proteins. The HMG proteins (80 μg), partially purified protein kinase NII (50 μl) and 20 μM [γ-^{32}P]ATP (10 μCi) were incubated at 30°C for 10 min in 0.5 ml total vol. which contained 12.5 mM $MgCl_2$ and 40 mM Tris–HCl (pH 7.2). After the reaction, the mixture was divided into two portions. (A) One portion (0.1 ml) was adjusted to 400 μl with the buffer solution, dialyzed against 0.01% SDS, 1 mM Na-phosphate (pH 7.2) for 18 h and lyophilized. (B) Another portion (0.4 ml) was adjusted to 5% perchloric acid by adding 60% solution to re-extract the HMG proteins. After centrifugation, the supernatant was dialyzed and lyophilized as above. The lyophilized proteins were dissolved in 40 μl 10% (w/v) sucrose, 0.15 M mercaptoethanol, and let stand at 30°C for 3 h. Samples of 20 μl each were subjected to SDS–polyacrylamide gel electrophoresis. The gel system was that of Laemmli except that the final concentrations of acrylamide and bis-acrylamide were 15% and 0.4%, respectively. After the electrophoresis, the gel was stained with Coomassie brilliant blue, destained and dried on filter paper (A). The dried gel was then subjected to autoradiography on a X-ray film (B). Samples are of whole reaction mixture (1), and HMG proteins re-extracted from the mixture (2). The position (R_F) of HMG 14 was determined under the same electrophoretic conditions employing a sufficient amount of HMG proteins.

The proteins in the histone preparation which were phosphorylated by NII kinase represent a set of non-histone proteins that are coextracted with histones in 0.25 M HCl [12]. In any event, fig.1 demonstrates that protein kinase NII highly phosphorylated the HMG proteins.

In fig.2, HMG proteins were compared with the other non-histone proteins in terms of relative substrate specificity for NII enzyme. The non-histone proteins were prepared from a 0.4 M NaCl nuclear extract by successive passages through a DEAE–Sephadex and a phosphocellulose columns. DEAE–Sephadex chromatography removed NII kinase from the nuclear extract, and the following phosphocellulose removed NI kinase. As seen in fig.2, both the HMG proteins and non-histone proteins served as substrate for NII enzyme, whereas a control substrate, bovine serum albumin, was not phosphorylated at all. When compared with non-histone proteins, the substrate efficiency of HMG proteins was remarkable: after 15 min incubation [^{32}P]phosphate incorporation into the HMG proteins was 5.5-fold higher than that into the non-histone proteins. This result indicates that the HMG proteins are a good substrate for protein kinase NII.

Fig.3 shows an autoradiogram of the [^{32}P]HMG

proteins which were phosphorylated by NII kinase. After incubation of the HMG proteins with [^{32}P]-ATP and the partially purified protein kinase NII, the reaction mixture, or the HMG proteins which were re-extracted from the mixture, were subjected to SDS–polyacrylamide gel electrophoresis. As seen on the stained gel (fig.3A), the HMG protein preparation contained, besides HMG proteins, Hl histones. The band corresponding to HMG 14 was not clearly discerned because of its low content in the original preparation and partly because of its possible degradation during the incubation. Upon examination of the [^{32}P]proteins on the autoradiogram (fig.3B), it is evident that HMG 1 and HMG 2 were hardly phosphorylated by protein kinase NII. In contrast to this, the band corresponding to HMG 17 was highly phosphorylated: the stained band and ^{32}P-labelled band were found not only at completely identical positions, but were also shown to bear a close resemblance in their shapes, although the ^{32}P-labelled band was wider due to longer exposure. Besides HMG 17, two other ^{32}P-labelled bands were seen; one at the major subfraction of H1 histone (H1A) and the other at the position of HMG 14. Since there was no recognizable stained band corresponding to HMG 14, we are not certain whether the latter ^{32}P-labelled band was actually attributable to HMG 14. However, this assignment seems highly possible because:

(i) The protein of this ^{32}P-labelled band was recovered from the reaction mixture under the conditions for extraction of HMG proteins;
(ii) The migration position (R_F) of the ^{32}P-labelled band coincided with that of HMG 14 on SDS gel;
(iii) HMG 14 has a high sequence similarity to HMG 17 [13] and is released from the active chromatin by nuclease digestion in a similar manner as HMG 17 [14,15].

Point (iii) suggests the similarity of the biological functions of HMG 14 and HMG 17 in chromatin, and supports the possibility that HMG 14 undergoes phosphorylation reaction in the same way as HMG 17.

4. Discussion

This study demonstrated that protein kinase NII preferentially phosphorylated the HMG proteins (fig.2), and that the phosphorylated proteins were HMG 17 and a protein which was probably HMG 14 (fig.3). Interestingly, NII kinase did not phosphorylate HMG 1 nor HMG 2 at all (fig.3). Furthermore, protein kinase NI was shown to hardly phosphorylate the HMG proteins (fig.1).

In addition to the phosphorylation, other post-synthetic modifications of HMG proteins have been recently reported: HMG 1, HMG 2 and their analogous protein from avian erythrocytes (HMG-E) are acetylated at ϵ-NH_2 groups of specific lysine residues [16,17]. HMG 1 and HMG 2 are also methylated at an arginine residue(s) [18]. Furthermore, H6 protein, a trout HMG protein which is an analog of HMG 17, has been documented to be ADP-ribosylated at a specific glutamic acid residue [19]. Thus, the HMG proteins undergo various post-synthetic modifications; acetylation, methylation, ADP-ribosylation and phosphorylation.

Although the function of the HMG proteins is not fully elucidated at present, the phosphorylation modification might regulate the functional activity of HMG proteins. Since they are enriched in the actively transcribing chromatin regions [2–7,14,15] and are likely to be structural proteins as suggested from their high abundance in the cell nucleus [1], they are thought to organize the chromatin structure necessary for gene transcription. On the other hand, it has been suggested that phosphorylation of nuclear non-histone proteins is involved in the regulation of gene transcription. In fact, changes in gene activity have been strongly correlated in various cells with an alteration in the phosphorylation of non-histone proteins [8]. Therefore, the phosphorylation of HMG protein(s) is expected to play an important role in the regulation of template activity of the active chromatins.

In contrast to NII kinase, NI kinase did not appreciably phosphorylate the HMG proteins (fig.1). This result demonstrates the different substrate specificity of NI and NII kinases, i.e., these enzymes act on different chromosomal proteins in the cell nucleus.

Since the phosphates incorporated into the HMG proteins were acid-stable (not shown), these phosphates are considered to be attached to hydroxyl groups of serine or threonine residues. The primary structures of HMG 14 and HMG 17 have been determined with calf thymus proteins [13,20]. Since there are considerable similarities in the sequence of HMG proteins from diverse sources (trout and calves [19]), we can assume that the primary structures of rat and calf HMG proteins are very similar. Based on this

assumption, the possible phosphorylation sites (serine and threonine) in HMG 17 consist of only two serines and one threonine [20]. The serines are at positions 24 and 28, and threonine at 76. Since the threonine residue is replaced by alanine in trout HMG 17 (H6 protein) [19], this threonine may not be a physiologically important site for phosphorylation. Therefore, it seems very likely that the phosphorylation of HMG 17 by protein kinase NII occurs at serine 24 and/or 28. If this is correct, then it is highly possible that HMG 14 undergoes a similar phosphorylation because an identical sequence consisting of 8 amino acid residues around these serines is also found in HMG 14 [13]. H6 protein from trout also possesses the same sequence [19].

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