IN VITRO PHOSPHORYLATION OF HISTONES H5, H2A, H2B AND OF THE DIMER H2A–H2B BY A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM RAT PANCREAS

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1. Introduction

Histone phosphorylation is a post-synthetic modification involved in the regulation of metabolic process and genetic expression [1,2] and associated with changes in chromatin structure [3].

All histones are phosphorylated but lysine-rich histones are the most active substrates for cyclic AMP-dependent protein kinases [4]. Phosphorylation of lysine-rich histone (H1) has been correlated with the rate of cell division in different rat and mouse tumors [5]. Phosphorylation is more extensive in rapidly growing cells than in non-dividing tissue. Phosphorylation is also related to cell maturation, for example, to erythrocyte maturation [6] during which time histone H5 is synthesized.

The phosphorylation of the histones with protein kinases isolated from different mammalian tissues: brain, liver, testis and thymus, takes place at specific sites of the amino acid sequences, generally located in highly basic regions which are major sites of interaction with DNA [7]. Therefore phosphorylation can modulate the histone–DNA interactions.

This paper deals with the in vitro phosphorylation of histones by a cyclic AMP-dependent protein kinase isolated from rat pancreas. Histones from various sources were used: H2B from rat thymus, H2A (variant α) from rat chloroleukemia; the complex H2A–H2B from calf thymus; H5 from chicken erythrocyte.

In rat chloroleukemia, histone H2A exhibits a polymorphism depending on the nature of the amino acid residues at positions 16 and 99 [8]. Three molecular species of H2A were identified in this tumoral tissue: H2A-α (serine 16 and lysine 99); H2A-β1 (threonine 16 and arginine 99); H2A-β2 (serine 16 and arginine 99). The variant α which accounts for 60% of the total H2A is of special interest because of a potential site of phosphorylation at serine residue in position 16.

On the other hand, the dimer H2A–H2B is known to be a constitutive element of the nucleosome [9] in the chromatin structure. It was therefore attractive to determine if the sites of phosphorylation were identical in the complex and in the individual histones.

The phosphorylated histones were hydrolysed with trypsin. The ^{32}P-labelled peptides were isolated and the sites of phosphorylation determined. Four sites of phosphorylation were identified in rat thymus histone H2B: two major, at serine residues 32 and 36, and two minor, at serine residues 87 and 91 [10].

Two sites of phosphorylation were identified in rat chloroleukemia histone H2A-α at serine residues 1 and 19. The serine residue at position 16 was not phosphorylated.

Since sequences of rat and calf histones H2A differ only by one conservative change at position 16 [8] Ser (rat) ⟶ Thr (calf) which is not a phosphorylation site and since rat and calf histones H2B have identical primary structures [10], the histone complex H2A–H2B was prepared from calf thymus chromatin in order to get a larger amount of substrate for our phosphorylation experiments.

When the dimer H2A–H2B was assayed, phosphorylation took place at the same serine residues and to the same extent as in the isolated histones.

In chicken erythrocyte histone H5, four phosphorylated sites were identified: at serine residues 22, 29, 145 and 166 [11].

Abbreviation: TPCK, Tos-Phe CH_2Cl, *N*-tosyl phenylalanine chloromethyl ketone

The major phosphorylation sites in all the assayed histones are generally located within predicted β-turns [12,13] and in the basic region of the proteins, which is involved in electrostatic interactions with the phosphate groups of the DNA.

2. Materials and methods

2.1. *Preparation of ^{32}P-labelled histones*

The variant α of rat chloroleukemia histone H2A was obtained by ion-exchange chromatography on Biorex 70 as in [8].

Rat thymus histone H2B was isolated by preparative electrophoresis on polyacrylamide slab gel [14]. The calf histone complex H2A–H2B was isolated from histone fraction F2b [15] by ion-exchange chromatography on Biorex 70 as in [16].

Chicken erythrocyte histone H5 was prepared as in [17].

Histones were phosphorylated by a cyclic AMP-dependent protein kinase isolated from rat pancreas. The catalytic subunit of this protein kinase was obtained, in a highly purified state by chromatography on CM–Sepharose [18].

About 20 mg of each histone (H2A-α, H2B, H5) and 40 mg of the dimer H2A–H2B were dissolved in 30 mM Tris–HCl (pH 7.8) containing 12 mM $MgCl_2$; histone was 0.5 mg/ml. The mixture was then incubated for 24 h at 30°C with the purified enzyme (1–1.5 μg/ml) in the presence of 0.4 mM [γ-^{32}P]ATP. The presence of an equimolar histone complex in these experimental conditions was assessed by circular dichroism spectroscopy (fig.1). Incubation was stopped by cooling (4°C) and the incubate was dialyzed against deionized water (2 × 3 l) for 18 h and lyophilized.

2.2. *Enzymatic cleavages of labelled histones and identification of the phosphorylation sites*

Tryptic hydrolysis: ^{32}P-labelled histones and dimer H2A-H2B were hydrolyzed for 2 h at pH 8.0 and 37°C in 0.1 M *N*-methyl morpholine with TPCK-treated trypsin (Worthington) using an enzyme-to-substrate ratio of 1:50 (w/w). The hydrolysis was stopped by lowering to pH 3.5 with formic acid.

Pepsin cleavage of histone H5: Alternatively, the phosphorylated histone H5 was dissolved in 5% formic acid and hydrolyzed for 2 h at 37°C with pepsin using an enzyme-to-substrate ratio of 1:50 (w/w) [11]. Hydrolysis was stopped by freezing. Fractionation of peptic peptides was performed on Sephadex G-50 fine (column 150 × 2.5 cm) equilibrated and eluted with 0.01 M HCl. The C-terminal peptide (peptide Px, 96 residues) was isolated and submitted to tryptic hydrolysis as above.

An aliquot was taken from each hydrolysate for peptide mapping on Whatman 3 MM paper as in [19].

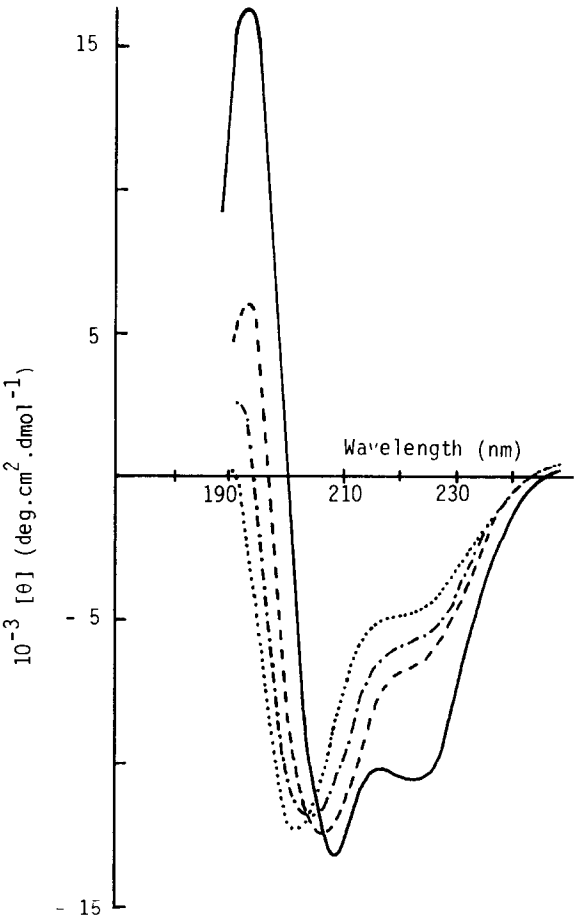

Fig.1. Circular dichroism spectra of calf thymus histones H2A (––), H2B (. . .), of a hypothetical non-interacting equimolar H2A/H2B mixture (·–·) and of the dimer H2A–H2B (——). Histones and dimer were dissolved in 30 mM Tris–HCl (pH 7.8) containing 12 mM $MgCl_2$ in the presence of 0.4 mM ATP. Histone was 0.5 mg/ml and measurements were made using a Jobin-Yvon dichrograph R. J. Mark III with a 0.1 mm pathlength cell. The dichroism spectrum of the dimer H2A–H2B is characteristic of a true complex namely by the $[\theta]_{222}$ value which accounts for – 10650°. This value agrees with that of – 11700° found [30] for the dimer H2A–H2B in 25 mM potassium phosphate (pH 7.0).

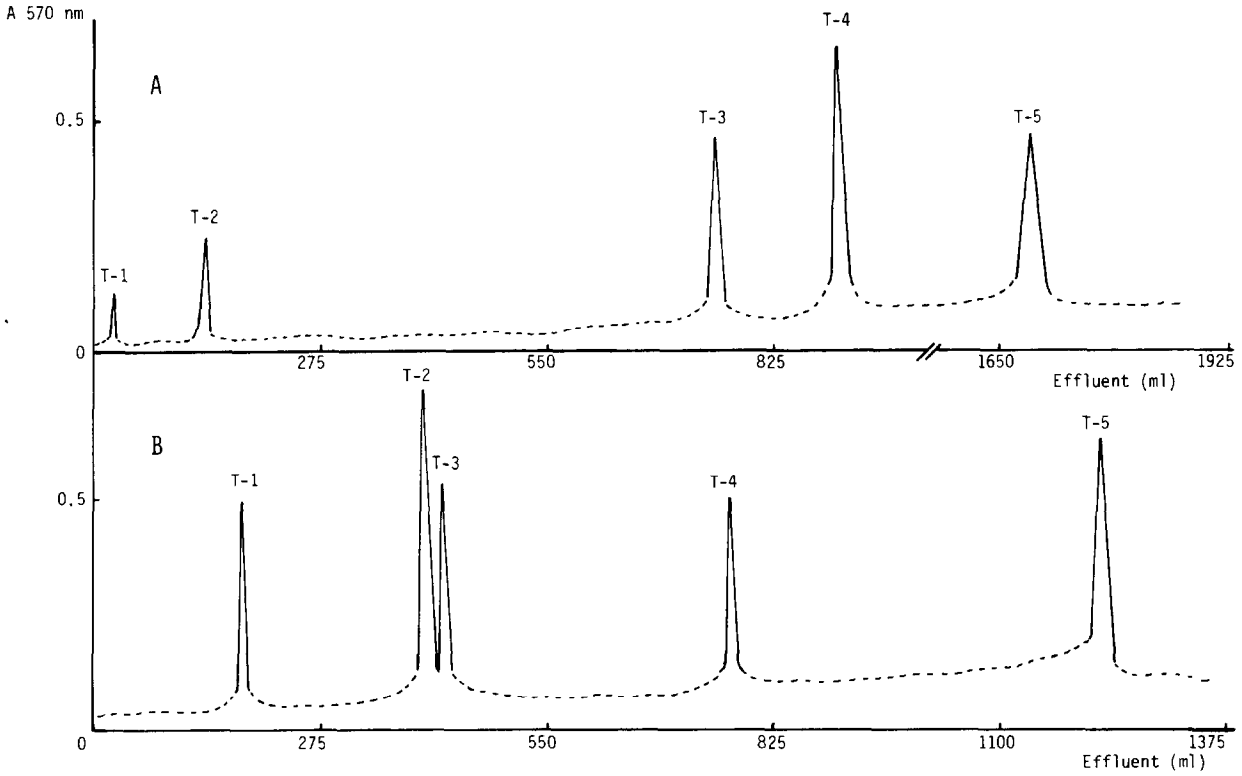

Fig.2. Elution diagrams of phosphorylated tryptic peptides, fractionated on a Chromobeads P column. Peptides were eluted with pyridine–formate and pyridine–acetate buffers used in a pyridine concentration and pH gradient [20]. Peptides were designated by the symbol T and numbered according to their elution volume of the Chromobeads P column. (A) Phosphorylated complex H2A–H2B; (B) phosphorylated histone H5 .

The tryptic hydrolysates were fractionated by ion exchange chromatography on a Chromobeads P column with pyridine formate and pyridine acetate buffers [20]. Fractions of 5.5 ml were collected at 33 ml/h flowrate. Elution of the peptides was monitored automatically by ninhydrin reaction. The Sakaguchi reaction was specifically used for the detection of the amino terminal peptide *N*-acetyl-Ser–Gly–Arg of histone H2A.

Fractions containing peptides were pooled, evaporated and taken into 1 ml 10% acetic acid. The radioactivity was then measured on an aliquot (5 μl) of each peptidic fraction after adding 2 ml Bray solution [21]. Fig.2 shows the elution diagrams of phosphorylated tryptic peptides from the dimer H2A–H2B (A) and from histone H5 (B).

The radioactive peptides were located on tryptic fingerprints by radioautography, using Kodirex film (Kodak). When necessary, purification of peptides was achieved by preparative paper chromatography or paper electrophoresis as in [19].

Amino acid composition of each phosphopeptide was determined after hydrolysis in 6 M HCl for 24 h at 110°C under vacuum. Under these conditions, the *O*-ester linkage was completely hydrolyzed [22].

The phosphorylated sites were identified by sequencing the phosphopeptides by manual Edman degradation and measuring the radioactivity after each cycle involving the elimination of a serine residue.

3. Results and discussion

The radioautography of the peptide map of the tryptic hydrolysate of the individual histones and of the dimer H2A–H2B labelled with [^{32}P]phosphate showed 2 labelled peptides in histone H2A-α, 4 in histone H2B and 5 in the complex H2A–H2B and in histone H5.

Structural studies of the phosphorylated tryptic

Table 1
Sites of in vitro phosphorylation of histones

Histones		Sequences of ^{32}P-labelled tryptic peptides	Amounts of phosphorylation[a]
Dimer H2A-H2B	T-1	N-acetyl-Ser(P)-Gly-Arg (1) (from H2A)	25
	T-2[b]	(Lys-Arg)-Ser(P)-Thr-Ile-Thr-Ser(P)-Arg (85, 87, 91) (from H2B)	4
	T-3	Ser-Arg-Ser-Ser(P)-Arg (19) (from H2A)	25
	T-4	(Lys)-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys (34, 36) (from H2B)	13
	T-5[c]	Lys-Arg-Ser(P)-Arg (32) (from H2B)	33
H5	T-1	Ser(P)-Arg (145)	24
	T-2	(Arg-Arg)-Ser(P)-Ala-Ser-His-Pro-Thr-Tyr-Ser(P)-Glu-Met-Ile-Ala-Ala-Ala-Ile-Arg (20, 22, 29)	20
	T-3	Lys-Ala-Ser(P)-Lys (166)	17
	T-4	Lys-Ala-Ser(P)-Lys-Ala-Lys-Lys (166)	13
	T-5	Lys-Lys-Ser(P)-Arg (145)	26

[a] The amounts of phosphorylation are expressed in % of the total amount of [^{32}P]phosphate incorporated into the proteins

[b] Residues in parentheses are not included in the tryptic peptides. They are written to show that the phosphorylation sites are placed in a sequence B–X–Ser(P) where B is a basic amino acid, X any amino acid

[c] In the dimer H2A–H2B, the bond Arg_{31}–$Ser(P)_{32}$ is not cleaved: this accounts for the presence of 5 radioactive spots (2 from histone H2A and 3 from histone H2B) instead of 6, on the radioautography of the peptide map whereas in histone H2B the cleavage of this bond generates peptide $Ser(P)_{32}$–Arg_{33} [10]

peptides isolated by chromatography on Dowex-50 allowed us to identify the sites of phosphorylation in each protein (table 1).

Two serine residues (Ser_1 and Ser_{19}) were phosphorylated in rat chloroleukemia histone H2A-α. The presence of a phosphate group on Ser_{19} completely inhibits the cleavage of the Arg_{17}–Ser_{18} bond by trypsin and results in the peptide:

$$Ser_{16}(P)\text{–}Arg\text{–}Ser\text{–}Ser\text{–}Arg_{20}$$

We must emphasize that the change Thr → Ser at position 16 of the amino acid sequence of rat histone H2A by comparison with the homologous calf histone, does not introduce a third site of phosphorylation in histone H2A.

Ser_1 was also found phosphorylated in vivo in trout testis [23] and rat liver [24] histone H2A.

On the other hand, Ser_{19} was the only site of in vitro phosphorylation identified in calf thymus histone H2A phosphorylated with the catalytic subunit of the cyclic AMP-dependent histone kinase isolated from pig brain [25].

Four sites of phosphorylation were identified in rat thymus histone H2B: Ser_{32} and Ser_{36} as major sites; Ser_{87} and Ser_{91} as minor sites [10]. The incomplete cleavage by trypsin of the bond Arg_{31}–$Ser_{32}(P)$ generated peptide Lys_{30}–Arg–$Ser_{32}(P)$–Arg_{33} together with peptide $Ser_{32}(P)$–Arg_{33} and consequently accounted for the presence of two radioactive spots corresponding to [^{32}P]Ser_{32} on the peptide map of histone H2B.

When the dimer H2A–H2B was phosphorylated, the presence of a true dimer in the experimental conditions of phosphorylation was ascertained by circular dichroism spectroscopy (fig.1). Increase of α-helix content typical of histone–histone interaction is seen at 222 nm by comparison with individual histone spectra.

In the dimer H2A–H2B, the sites of phosphorylation were found identical to those determined in the individual histones. These sites are located outside or at the edge of the structured apolar regions (18–127 of H2A and 32–125 of H2B) which strongly interact together to form the histone complex H2A–H2B (M. C., unpublished). The highly basic N-terminal regions of H2A and H2B are not involved in dimer formation and are major sites of interaction of DNA.

Clearly, the phosphorylation sites of H2A and H2B are still accessible to the kinase when H2A and H2B interact together.

Five radioactive peptides were identified in the tryptic hydrolysate of chicken erythrocyte histone H5. One peptide corresponding to the sequence 22–37 of the protein contained two sites of phosphorylation (Ser_{22} and Ser_{29}). The third serine residue present in the peptide at position 24 was not phosphorylated. The amount of radioactivity measured in this peptide represents ~20% of the total labelling of the protein. The remainder of the radioactivity is equally distributed between Ser_{145} and Ser_{166} in the highly basic C-terminal half of the histone H5.

Due to incomplete cleavage by trypsin of the susceptible bonds adjacent to the phosphorylated sites, the labelled Ser_{145} and Ser_{166} were each identified in two different peptides (table 1). However, since the peptide Ser–Arg can be generated by tryptic hydrolysis of the Lys–Ser bond present 3 times in the amino acid sequence of histone H5 (residues 40–42; residues 144–146; residues 161–163), it was necessary to ascertain the position of the peptide $Ser(P)$–Arg in the sequence of the protein. Phosphorylated histone H5 was therefore hydrolysed by pepsin in order to yield the C-terminal half of the protein (residues 94–189) by specific cleavage of the Phe_{93}–Arg_{94} bond. The tryptic digestion of this peptide generated peptides:

$$Lys\text{–}Lys\text{–}Ser_{145}(P)\text{–}Arg; \quad Lys\text{–}Ala\text{–}Ser_{166}(P)\text{–}Lys;$$

$$Lys\text{–}Ala\text{–}Ser_{166}(P)\text{–}Lys\text{–}Ala\text{–}Lys\text{–}Lys$$

No peptide $Ser(P)$–Arg was found in the tryptic digest of the peptic peptides originating from the amino-terminal half of the histone H5.

The sites of phosphorylation of chicken histone H5 identified here are different from those found in the homologous protein from pigeon erythrocyte [26]. The in vitro phosphorylation of pigeon histone

H5 with a pig brain protein kinase occurred at Ser_{45} (or Ser_{46}), Ser_{49}, Ser_{91} (or Ser_{92}) and at a serine or threonine residue in the carboxy-terminal half of the molecule. The protein kinase from pig brain seems therefore to have a specificity different from that of the protein kinase from rat pancreas. This difference of specificity has already been pointed out in the phosphorylation of histone H2B [10].

However, the in vitro phosphorylation sites of histone H5 are different from those of the in vivo phosphorylation [27]. In vivo, Ser_3 and Ser_7 are strongly ^{32}P-labelled to the same specific activity. In the carboxy-terminal part of the molecule (residues 121–189), the two serine residues phosphorylated in vivo have not been identified with certainty but they appear to be different from the in vitro sites (Ser_{145} and Ser_{166}).

Our results show that the phosphorylation of histone H5 occurs mainly in the strongly basic carboxy-terminal region of the molecule where 80% of the total amount of radioactivity are incorporated. The remaining ^{32}P-label is found in the slightly basic N-terminal part of the molecule at the edge of the globular region of the protein (residues 23–99) [28].

However, even if the specificity is different from one kinase to another, the sites of phosphorylation in histone H5 are distributed in vitro or in vivo, in two distinct regions of the protein: the basic carboxy-terminal region and the apolar amino-terminal region.

Phosphorylation of the histones H2A, H2B and H5 by the cyclic AMP-dependent protein kinase isolated from rat pancreas occurs generally on serine residue located in a sequence B–X–Ser where B is Lys or Arg and X any amino acid (table 1). However, phosphorylation of Ser_1 in H2A, Ser_{91} in H2B and Ser_{29} in H5 does not meet with this code, but it must be stated that Ser_{91} in H2B and Ser_{29} in H5 are minor phosphorylation sites. The phosphorylated Ser_{32} in H2B and Ser_{145} in H5 on the one hand and Ser_{19} in H2A and Ser_{166} in H5, on the other hand, are located in strongly homologous sequences, respectively.

The recognition of specific amino acid sequences by the protein kinase is completed by that of a specific secondary structure. Indeed, all the phosphorylation sites are located within regions predicted as β-turns [12,13].

Phosphorylation essentially takes place in the highly basic regions of the histones involved in electrostatic interactions with the phosphate groups of DNA. The 3 minor sites (Ser_{87} and Ser_{91} in H2B, and Ser_{29} in H5) which are located inside the structured apolar regions of the proteins [28,29], are brought to the surface of the protein by a β-turn structure and therefore are accessible to the protein kinase [13]. The major sites of phosphorylation are placed outside the organised apolar regions of the proteins [29].

The fact that Ser_1 and Ser_{19} in H2A and Ser_{32} and Ser_{36} in H2B are phosphorylated in the histone complex H2A–H2B is in conformity with that determination.

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