PHOSPHORYLATED SITES OF CALF THYMUS HISTONE H2B BY ADENOSINE 3',5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM SILKWORM*

Eikichi Hashimoto, Masao Takeda and Yasutomi Nishizuka

Department of Biochemistry
Kobe University School of Medicine, Kobe, Japan

Koei Hamana and Koichi Iwai

Institute of Endocrinology, Gunma University, Maebashi, Japan

Received July 21,1975

SUMMARY: Calf thymus histone H2B was ^{32}P-labelled by incubation with [γ^{32}P]ATP and adenosine 3',5'-monophosphate-dependent protein kinase from silkworm pupae. Three major radioactive tryptic phosphopeptides were isolated by a series of column chromatographies and analyzed for their amino acid compositions. Comparison of the data with the known primary structure of the histone revealed their amino acid sequences as Lys-Glu-Ser^{36}-Tyr-Ser^{38}-Val-Tyr-Val-Tyr-Lys, Lys-Arg-Ser^{32}-Arg and Ser^{32}-Arg. Chymotryptic digestion of the first tryptic phosphopeptide produced quantitatively radioactive Lys-Glu-Ser^{36}-Tyr. Eighty five per cent of the initial acid-precipitable phosphate was recovered at Ser-32 (32%) and Ser-36 (53%).

For clarifying the involvement of primary structure in determining substrate specificity of cyclic AMP[1/]-dependent protein kinase, the data on the amino acid sequences in the vicinity of the sites phosphorylated by the enzyme have been accumulated with various substrates such as histone H1 (1,2), histone H2A (2), phosphorylase kinase (3), pyruvate kinase (4), myelin basic protein (5) and reduced carboxymethylated maleylated lysozyme (6). Recently, Farago et al. (7) isolated a major tryptic phosphopeptide

* This investigation has been supported in part by the research grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Toray Science Foundation and Scientific Research Fund of the Ministry of Education of Japan.

1/ The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; cyclic GMP, guanosine 3',5'-monophosphate.

from calf thymus histone H2B which was phosphorylated by cyclic AMP-dependent protein kinase partially purified from human tonsillar lymphocytes, and identified as Lys-Glu-Ser^{36}-Tyr-Ser^{38}-Val-Tyr-Val-Tyr-Lys. They proposed either Ser-36 or Ser-38 as the major site of phosphorylation. More recently, Shlyapnikov et al. (2) also isolated the same tryptic phosphopeptide from calf thymus histone H2B which was phosphorylated by pig brain cyclic AMP-dependent protein kinase. In this communication, we conclusively identified Ser-36 as one of the major sites of phosphorylation in calf thymus histone H2B by cyclic AMP-dependent protein kinase from silkworm pupae; Lys-Glu-Ser^{36}(P)-Tyr was quantitatively obtained after chymotryptic digestion of the tryptic phosphopeptide mentioned above. Furthermore, we defined Ser-32 as another major site of phosphorylation by the protein kinase.

Histone H2B was prepared from calf thymus by the method of Oliver et al. (8). Cyclic AMP-dependent protein kinase employed for the present study was purified from the extract of silkworm *(Bombyx mori)* pupae by a slight modification of the method described by Nishiyama et al. (9). The crude extract was prepared by homogenizing the frozen pupae with 4 volumes of 4 mM EDTA containing 5 mM magnesium acetate and 6 mM 2-mercaptoethanol (pH 7.0), followed by centrifugation for 20 min at 27,000 x g. The extract was subjected to pH 4.8 treatment in a manner described by Miyamoto et al. (10), and all subsequent operations (calcium phosphate-cellulose and DE52 column chromatographies and gel filtration) were carried out as described by Nishiyama et al. (9) except that 1) calcium phosphate-cellulose column chromatography was repeated twice to remove cyclic GMP-dependent protein kinase completely; 2) Sephadex G-150 was used instead of Sephadex G-200; and 3) magnesium acetate and EDTA were omitted from all buffers. Upon gel

filtration on a Sephadex G-150 column cyclic AMP-dependent protein kinase was resolved into two, one major and one minor, peaks; the major peak (molecular weight, 1.8×10^5) was used for the present study. By this procedure the enzyme was purified about 100-fold from the original crude extract and the preparation was free of cyclic GMP-dependent protein kinase as well as of endogenous phosphate acceptor protein under the conditions employed for histone H2B phosphorylation.

Histone H2B (200 mg) was phosphorylated for 3 hr at 30°C in 300 ml of the reaction mixture containing 20 mM Tris-HCl at pH 7.5, 20 mM magnesium acetate, 4×10^{-5} M [γ-^{32}P]ATP (17.5×10^3 cpm per nmole), 10^{-7} M cyclic AMP, and the enzyme (36 mg protein). Under these conditions, the incorporation of phosphate reached maximum and 0.83 mole of ^{32}P was incorporated per mole of histone H2B. Most of the radioactivity incorporated into the histone was shown to be associated with phosphoserine after acid hydrolysis. The reaction was stopped by freezing. After being lyophilized, the residue was dissolved in 30 ml of H_2O and 100% (w/v) trichloroacetic acid was added to a final concentration of 10%. After standing for 20 min in ice, the precipitate was collected by centrifugation for 10 min at 15,000 x g, and washed twice with 10 ml of 10% trichloroacetic acid, once with acetone-HCl (0.05 ml conc HCl in 100 ml acetone) and once with acetone. After being dried, the radioactive histone H2B was extracted from the precipitate three times with 20 ml (total) of 0.25 N HCl by homogenizing in a Dounce homogenizer followed by centrifugation for 10 min at 15,000 x g. The histone was precipitated by adding 8 volumes of acetone and standing overnight at -20°C. The precipitate was collected by centrifugation for 10 min at 15,000 x g and washed once with 10 ml of cold acetone and dried under vacuum. These

procedures minimized the enzyme protein which contaminated the radioactive histone H2B preparation finally obtained.

The radioactive histone preparation (142 mg protein; 9,500 nmoles ^{32}P) was digested with trypsin (Worthington, Code TRTPCK) (Trypsin-histone=1:50) for 4 hr at 37°C in 27 ml of 0.1 M ammonium carbonate buffer at pH 8.5. After being lyophilized, the tryptic digests were taken up with 4 ml H_2O and subjected to gel filtration through a Sephadex G-15 column. As shown in Fig. 1, the radioactive phosphopeptides were resolved into two fractions (peak A and peak B), and the recovery was almost quantitative (7,400 nmoles ^{32}P in peak A; 2,000 nmoles ^{32}P in peak B). When peak A was subjected to SP-Sephadex column chromatography under the conditions given in Fig. 2, 7 radioactive peaks were obtained

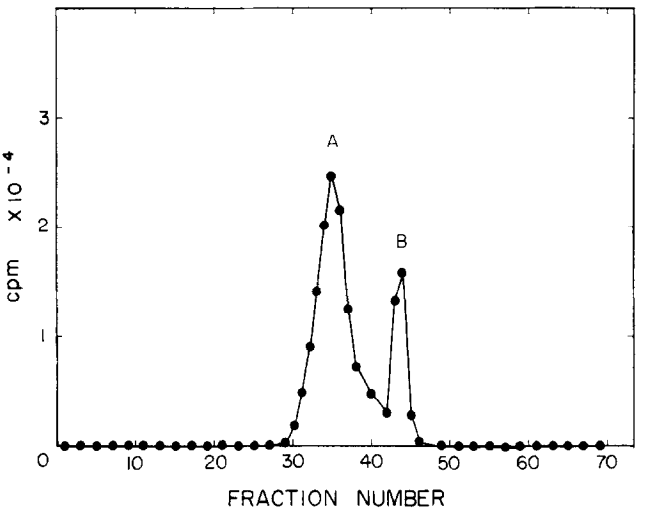

Fig. 1. Gel filtration of tryptic ^{32}P-peptides from phosphorylated histone H2B on a Sephadex G-15 column. A Sephadex G-15 column (1.7 x 112 cm) was equilibrated with 50% (W/V) acetic acid. Elution was performed with the same acetic acid solution at a flow rate of 11 ml per hr. Fractions of 3 ml each were collected. A 5 µl aliquot of each fraction was placed on an aluminum planchet, dried and measured for the radioactivity with a Nuclear Chicago Geiger Muller gas flow counter, model 4338. See text for other experimental details.

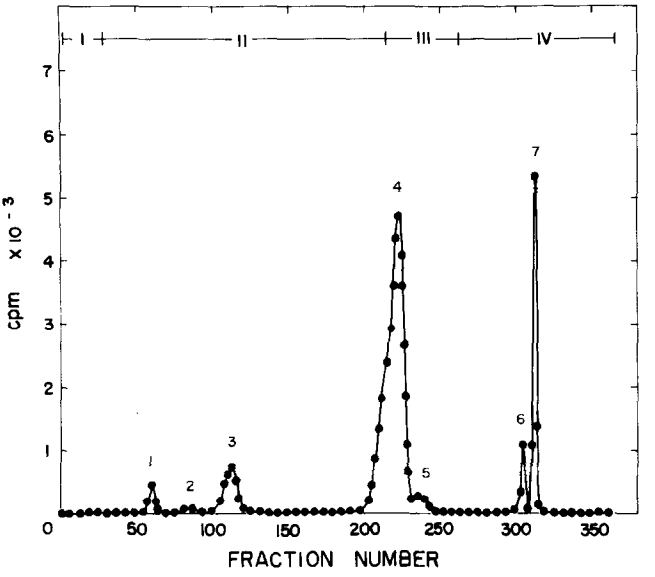

Fig. 2. SP-Sephadex column chromatography of peak A. Peak A (6,500 nmoles, ^{32}P) from a Sephadex G-15 column was lyophilized and taken up with 7.5 ml of 0.1 M pyridine-acetic acid buffer, pH 3.1 and applied to a SP-Sephadex column (2.2 x 50 cm) equilibrated with the same buffer. Elution was performed at a flow rate of 8.0 ml per hr with the following buffers; I, 140 ml of 0.1 M pyridine-acetic acid buffer, pH 3.1; II, a linear gradient (total volume 1,000 ml) formed from 0.1 M, pH 3.1 and 0.25 M, pH 3.25 pyridine-acetic acid buffer; III, 240 ml of 0.25 M pyridine-acetic acid buffer, pH 3.25; IV, a linear gradient (total volume 500 ml) formed from 0.25 M pyridine-acetic acid buffer, pH 3.25 and 8.25 M pyridine. Fractions of 5 ml each were collected. The radioactivity of a 10 µl aliquot of each fraction was measured for the radioactivity as described in the legend to Fig. 1.

---

as indicated, and more than 80% of the applied radioactivity was recovered in peaks 3, 4 and 7. Under analogous conditions peak B was resolved into two peaks, one major and one minor, as shown in Fig. 3 and more than 90% of the radioactivity was recovered in peak B_1. Peaks A_3, A_4, A_7 and B_1 were lyophilized separately and purified further; each sample (about 300 nmoles) was dissolved in 10 ml of 0.05 M ammonium bicarbonate buffer at pH 8.5 and applied to a QAE-Sephadex column (2.4 x 13 cm) equilibrated with the same buffer. After washing with 100 ml of the same buffer, elution was performed with a 700 ml-linear concentration gradient (50 mM

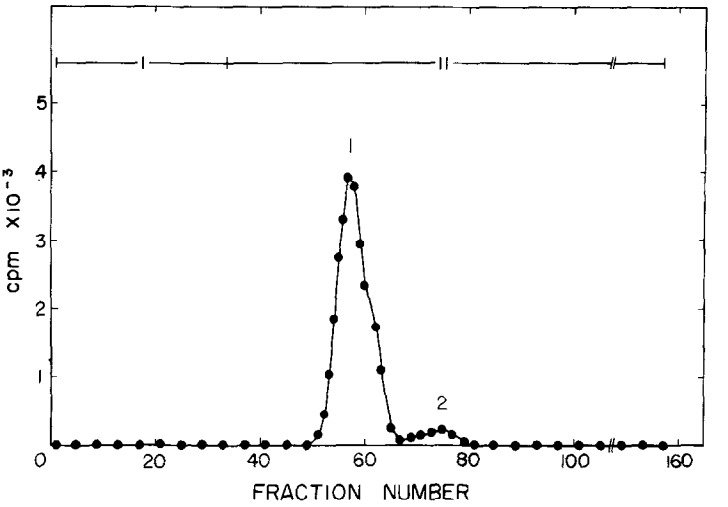

Fig. 3. SP-Sephadex column chromatography of peak B. Peak B (1,800 nmoles, ^{32}P) from a Sephadex G-15 column was applied to a SP-Sephadex column (2.2 x 30 cm) and eluted as described in the legend to Fig. 2 except that the following elution buffers were used; I, 170 ml of 0.1 M pyridine-acetic acid buffer, pH 3.1; II, a linear gradient (total volume 600 ml) formed from 0.1 M, pH 3.1 and 0.25 M, pH 3.25 pyridine-acetic acid buffer. For other experimental details see the legend to Fig. 2.

---

to 500 mM) of the same buffer, and fractions of 7 ml each were collected at a flow rate of 70 ml per hr. Peaks A_4, A_7 and B_1 were eluted as a sharp single peak at about 0.4 M, 0.06 M and 0.3 M ammonium bicarbonate buffer, respectively. Peak A_3 was eluted at about 0.55 M with a 700 ml-linear concentration gradient (500 mM to 1 M) of the same buffer. These tryptic phosphopeptides were lyophilized, dissolved in 2 ml of 0.2 N acetic acid, and was applied to a Sephadex G-10 column (1.7 x 106 cm) equilibrated with 0.2 N acetic acid. Elution was performed with 0.2 N acetic acid at a flow rate of 20 ml per hr. Each tryptic phosphopeptide appeared as a single peak. The samples (about 100 nmoles each) were hydrolyzed in 6 N HCl for 24 hr at 110°C in the presence of 4 mM phenol under reduced pressure and analyzed for amino acid composition with a Hitachi amino acid analyzer, model KLA 3B. Compari-

son of the amino acid compositions given in Table I with the known primary structure of H2B (11,12) revealed that the amino acid sequences of peaks A_3, A_4, A_7 and B_1 were Lys-Glu-$\overset{36}{\text{Ser}}$-Tyr-$\overset{38}{\text{Ser}}$-Val-Tyr, Lys-Glu-$\overset{36}{\text{Ser}}$-Tyr-$\overset{38}{\text{Ser}}$-Val-Tyr-Val-Tyr-Lys, Lys-Arg-$\overset{32}{\text{Ser}}$-Arg and $\overset{32}{\text{Ser}}$-Arg, respectively. Glycine found in peak A_3 (0.7 mole) and in peak A_7 (0.2 mole) was presumably a contaminant due to unknown technical reasons (Table I). The results indicated that both peaks A_3 and A_4 contained two seryl residues (Ser-36 and Ser-38) and one phosphate group. Therefore, peak A_4 (600 nmoles) was digested for additional 4 hr at 37°C with 0.5 mg of chymotrypsin (Worthington, Code CDS) in 1.6 ml H_2O (pH was adjusted to 7.8 with NH_4OH). The chymotryptic radioactive phosphopeptide (A_{4C}) was purified by QAE-Sephadex column chromatography followed by gel filtration on a Sephadex G-10 column as

Table I

Amino acid compositions of tryptic and chymotryptic phosphopeptides from histone H2B[1)]

Amino acid	Phosphopeptide				
	A_3	A_4	A_{4C}	A_7	B_1
Lys	0.8	2.0	0.9	0.9	
Arg				2.1	1.2
Ser[2)]	2.1	2.0	1.0	1.0	0.8
Glu	1.3	1.1	1.1		
Gly	0.7			0.2	
Val	1.0	1.8			
Tyr	1.8	3.0	1.0		
32p[3)]	0.9	1.1	1.0	0.9	1.0

1) Data was presented in molar ratio.
2) Corrected for 15% loss during hydrolysis.
3) Calculated from radioactivity.

described above; each time the phosphopeptide appeared as a single peak with an overall recovery of more than 80%. Then, the amino acid sequence of the chymotryptic phosphopeptide (A_{4C}) was identified as Lys-Glu-$\overset{36}{\text{Ser}}$-Tyr (Table I). The results indicated that one of the main phosphorylated sites of histone H2B was Ser-36.

Another main phosphorylated site of histone H2B was identified as Ser-32 from the amino acid sequence of peak A_7 (Lys-Arg-$\overset{32}{\text{Ser}}$-Arg) and that of peak B_1 ($\overset{32}{\text{Ser}}$-Arg). The tryptic hydrolysis of the peptide bond between Arg-31 and Ser-32 appeared to be partially protected by the phosphate attached to Ser-32. Under the present conditions the tryptic digestion of phosphorylated histone H2B produced Lys-Arg-$\overset{32}{\text{Ser}}$(P)-Arg and $\overset{32}{\text{Ser}}$(P)-Arg in the ratio of 1:2.2. The dipeptide of Ser(P)-Arg was produced from Lys-Arg-Ser(P)-Arg by extensive tryptic digestion as judged by paper chromatography followed by radioautography (data not shown). The amounts of phosphate incorporated into Ser-32 and Ser-36 were more than 32% and 53%, respectively, of the total phosphate incorporated into histone H2B. Therefore, more than 85% of the radioactivity was recovered at these two sites.

Shlyapnikov et al. (2) have isolated Lys-Gly-$\overset{14}{\text{Ser}}$(P)-Lys as one of the major tryptic phosphopeptides of the histone phosphorylated by pig brain enzyme. However, this phosphopeptide has not been obtained at least as a major tryptic phosphopeptide in the present studies. It may be noted that most of the serine residue phosphorylated by cyclic AMP-dependent protein kinase is in the sequence of X-Y-Ser, where X is lysine or arginine and the peptide bond between X and Y is strongly protected from tryptic digestion by the phosphorylation of the serine residue.

ACKNOWLEDGEMENT: Authors wish to express their thanks to Mrs. Sachiko Nishiyama and Miss Miwako Kuroda for their secretarial assistance.

REFERENCES

1. Langan, T.A. (1969) Proc. Nat. Acad. Sci. U.S.A., 64, 1276-1283.
2. Shlyapnikov, S.V., Arutyunyan, A.A., Kurochkin, S.N., Memelova, L.V., Nesterova, M.V., Sashchenko, L.P., and Severin, E.S. (1975) FEBS Letters, 53, 316-319.
3. Cohen, P., Watson, D.C., and Dixon, G.H. (1975) Eur. J. Biochem., 51, 79-92.
4. Hjelmquist, G., Andersson, J., Edlund, B., and Engström, L. (1974) Biochem. Biophys. Res. Commun., 61, 509-513.
5. Carnegie, P.R., Dunkley, P.R., Kemp, B.E., and Murray, A.W. (1974) Nature, 249, 147-150.
6. Bylund, D.B. (1974) Fed. Proc., 33, 1323.
7. Farago, A., Romhanyi, T., Antoni, F., Takats, A., and Fabian, F. (1975) Nature, 254, 88.
8. Oliver, D., Sommer, K.R., Panyim, S., Spiker, S., and Chalkley, R. (1972) Biochem. J., 129, 349-353.
9. Nishiyama, K., Katakami, H., Yamamura, H., Takai, Y., Shimomura, R., and Nishizuka, Y. (1975) J. Biol. Chem., 250, 1297-1300.
10. Miyamoto, E., Kuo, J.F., and Greengard, P. (1969) J. Biol. Chem., 244, 6395-6402.
11. Iwai, K., Ishikawa, K., and Hayashi, H. (1970) Nature, 226, 1056-1058.
12. Ishikawa, K., Hayashi, H., and Iwai, K. (1972) J. Biochem., 72, 299-326.