Volume 294, number 1,2, 104–108 FEBS 10461 December 1991

Identification of the phosphorylation sites of H2B histone by a catalytic fragment of p72^{syk} from porcine spleen

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Received 2 October 1991; revised version received 17 October 1991

Phosphorylated sites of calf thymus H2B histone were investigated with a catalytic fragment of 72 kDa protein-tyrosine kinase (p72^{syk}). Three of five tyrosine residues in H2B histone can be phosphorylated by this kinase. In this analysis, H2B histone was thoroughly phosphorylated in vitro with [γ-^{32}P]ATP and the kinase, and then digested with a lysylendopeptidase. The resulting radioactive phosphopeptides were separated by a reverse-phase column on high performance liquid chromatography. Subsequent sequential Edman degradation of the purified phosphopeptides revealed that ^{40}Y, ^{83}Y and ^{121}Y were phosphorylated. ^{121}Y is the major phosphorylated residue in H2B histone. No phosphorylation was detected in ^{37}Y and ^{42}Y. Although the consensus sequence was not defined from these analyses, our data suggest that higher-order structure(s) in addition to primary one may participate in recognition of H2B histone by this protein kinase.

p72^{syk}; Protein-tyrosine kinase; H2B histone; Phosphorylation

1. INTRODUCTION

Since the discovery of the protein-tyrosine kinases more than 30 protein-tyrosine kinases have been identified [1]. They are classified into a transmembrane receptor family and a nonreceptor one [2]. Compared to the transmembrane receptor type protein-tyrosine kinases, the physiological function of nonreceptor type protein-tyrosine kinases is not well understood. We have purified the nonreceptor type 40 kDa protein-tyrosine kinase from cytosolic fraction of porcine spleen [3,4]. Recently it has been clarified that this protein-tyrosine kinase was generated from the 72 kDa protein (p72^{syk}) by proteolysis and the structure of the cDNA was also revealed [5]. In order to know the mechanism of substrate recognition of this kinase, first we try to identify recognition sites of substrate proteins. We used calf thymus H2B histone as a model substrate, because this basic protein is one of the best substrates for this protein kinase in vitro [3,6].

The amino acid sequences around the phosphorylated tyrosine residues by a number of protein-tyrosine kinases have been reported [7–9] and it has been indicated that acidic amino acids are often located near the phosphate-accepted tyrosine residues [10,11]. On the other hand, it has been also suggested that the relatively lower specificity in phosphorylation of small peptide substrates by protein-tyrosine kinase has led to the idea of the importance of secondary and tertiary structure in the recognition of protein substrates [7,12]. In fact Tinker et al. have reported that β-turn of the substrates is important for the recognition by the protein-tyrosine kinase [13].

In this paper we clarified the phosphorylation sites of H2B histone by the catalytic fragment of p72^{syk} and neither acidic nor basic amino acids around tyrosine residues are important for the recognition by this kinase.

The sequence data of p72^{syk} will appear in the Genbank, the EMBL Data Library and the DNA Data Bank of Japan under the accession number M73237.

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2. EXPERIMENTAL

2.1. *Materials and chemicals*

H2B histone was prepared by the method of Hashimoto et al. [14], and was purified by a reverse-phase C18 column (0.39 × 15 cm, μBondasphere 5 μm C18-100Å, Waters) on high performance liquid chromatography (HPLC). Catalytic fragment of p72^{syk} from porcine spleen was employed as a catalyst [4,5] because we did not succeed in the purification of this kinase with the molecular mass of 72 kDa as yet. [γ-^{32}P]ATP was obtained from ICN. Lysylendopeptidase (Achromobactor lytics M497-1) was obtained from Wako Pure Chemicals. Other chemicals were purchased from commercial sources.

2.2. *Phosphorylation of H2B histone*

Phosphorylation of H2B histone was performed in 5 ml of reaction mixture containing 50 mM Hepes (pH 7.5), 200 μM [γ-^{32}P]ATP, 5 mM $MnCl_2$, 10 μM vanadate, 100 μg/ml phosphatidic acid, 100 nmol H2B histone and 40 kDa kinase. After incubation for 2 h at 30°C, the reaction was stopped by the addition of HCl at a final concentration

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of 10 mM. To remove [γ-^{32}P]ATP from the reaction mixture, the mixture was applied to Dowex-1 (300 μl) which was equilibrated with 10 mM HCl. Then the column was washed with 3 ml of 10 mM HCl to elute H2B histone. The eluate was lyophilized and dissolved in 1 ml of H_2O, and was applied to a reverse-phase C18 column on the HPLC and eluted at a constant flow rate of 1 ml/min, with a linear gradient of acetonitrile from 0% to 50% in 0.2% trifluoroacetic acid (pH 2.0) at room temperature. UV absorbancy at 210 nm and radioactivity were monitored. Radioactivity of ^{32}P was determined with an Aloka LSC-950 liquid scintillation counter by Cerenkov radiation. Phosphorylated H2B histone fractions were collected and concentrated by the lyophilizer. The concentrated materials were dissolved in 1.5 ml of 1 M Tris-HCl (pH 9.3) and were digested at 30°C for 24 h by lysylendopeptidase (lysylendopeptidase/H2B histone, $^1/_{250}$ (mol/mol). The mixture was applied to the reverse-phase C18 column on the HPLC again and eluted at the same condition described above. The radioactive phosphopeptides were further purified as indicated in each experiment.

2.3. *Sequential Edman Degradation*

Amino acid sequences of purified phosphopeptides were determined by automated Edman degradation using a gas-phase protein sequencer (Applied Biosystems, model 477A) equipped with an on-line reverse-phase chromatography system for identification of phenylthiohydantoin (PTH) amino acids.

3. RESULTS

After incubation for 2 h with 40 kDa kinase, 0.92 mol of phosphate was incorporated per mol of H2B histone as judged by the acid-precipitable radioactivity. Subsequent incorporation of radioactive phosphate was extremely slow, and the enzyme did not appear to be limited. After H2B histone was treated with lysylendopeptidase, the elution profile of phosphopeptides from reverse-phase column was shown in Fig. 1. The radioactive peaks in Fig. 1 were named from left side, peaks I, II, III, IV, V and VI, respectively. As peak I was completely passed through the reverse-phase column on HPLC, it was applied to a Sephadex G15 column (1.5 × 94 cm) for the purposes of removing Tris-HCl and

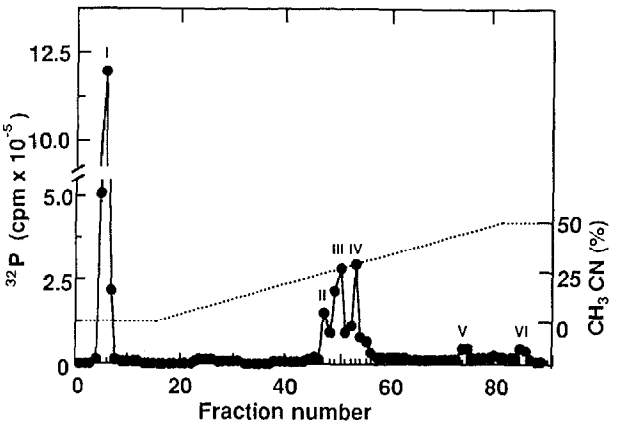

Fig. 1. Separation of radioactive phosphopeptides by a reverse-phase column on HPLC. Lysylendopeptidase-treated phosphopeptides were injected into a reverse-phase C18 column as described in section 2. Fractions of 0.5 ml each were collected and the radioactivity was determined. ●---● and ---, radioactivity of phosphopeptide and concentration of acetonitrile, respectively.

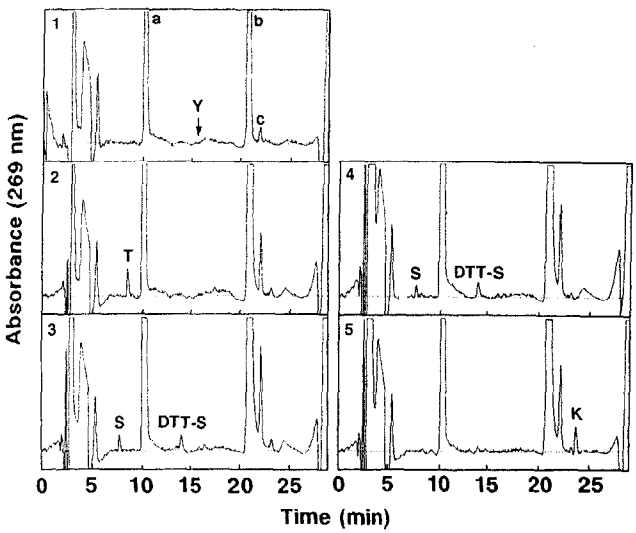

Fig. 2. Sequence analysis of peak I after separation by a reverse-phase column on HPLC. Phosphopeptide I was redissolved in 100% acetonitrile and analyzed with the gas phase sequencer. The figure shows reverse-phase chromatograms of PTH derivatives corresponding to the first 5 cycles. More than 50% of phenylthiohydantoin derivative of serine was detected as its dithiothreitol adduct (DTT-S) [21]. *Other abbreviations:* Y, PTH-tyrosine; T, PTH-threonine; S, PTH-serine; K, PTH-lysine; a, b and c are dimethylphenylthiourea, diphenylthiourea and diphenylurea, respectively.

purifying the radioactive peptide. A single radioactive peak was eluted from this gel filtration column. The peak fractions were collected, and applied to the reverse-phase column on HPLC again. The phosphopeptide in peak I could be slightly adsorbed to the reverse-phase column on HPLC after removing Tris-HCl. The result of sequence analysis of peak I was shown in Fig. 2. The sequence of peak I corresponded to ^{121}Y-T-S-S-K and ^{121}Y was identified as the phosphorylated site, because the peak of PTH derivative of phosphotyrosine was not detected in this sequencing system [15].

When peak II was further digested with lysylendopeptidase and applied to the same reverse-phase column, the radioactive peak was eluted at the position corresponding to that of peak I (data not shown). This result suggested that peak II was produced by incomplete digestion of peptides containing peak I.

In the next experiment, peak III in Fig. 1 was further purified by rechromatography on reverse-phase column. The peak III was lyophilized and dissolved in water, and was applied to a reverse-phase C18 column on HPLC and eluted with a linear gradient of acetonitrile from 0% to 21% in 10 mM sodium phosphate (pH 4.4). Then, the radioactive peak eluted at 18% acetonitrile in this buffer was collected, lyophilized and dissolved in H_2O. The radioactive peptide was reapplied to the reverse-phase C18 column and eluted with a linear gradient of acetonitrile from 0% to 30% in 0.2% trifluoroacetic acid (pH 2.0). The latter procedure was employed for removing the sodium phosphate before

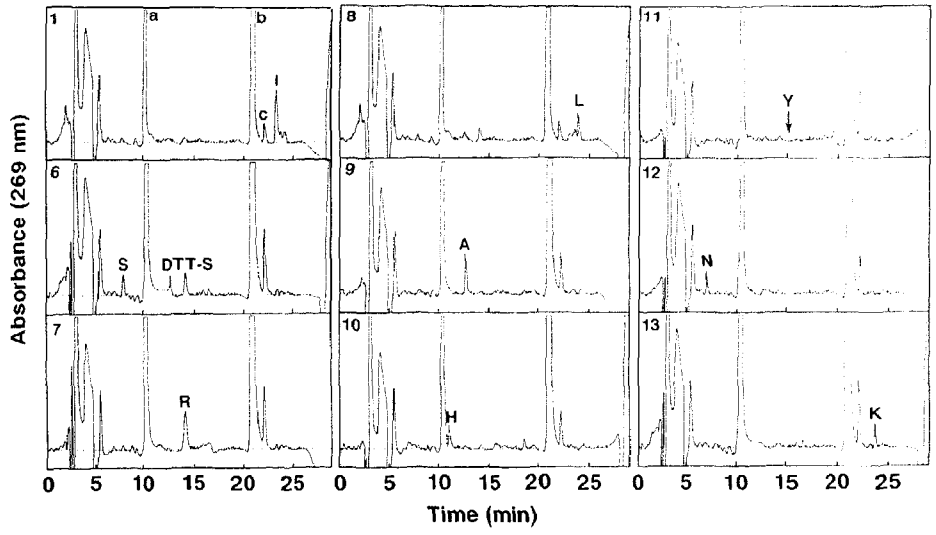

Fig. 3. Sequence analysis of peak III after rechromatography by a reverse-phase column of HPLC. Phosphopeptide III was redissolved in 100% acetonitrile and analyzed with the gas phase sequencer. The figure shows reverse-phase chromatograms of PTH derivatives corresponding to the first 13 cycles. Cycles 2–5 are not shown. *Abbreviations:* I, PTH-isoleucine; R, PTH-arginine; L, PTH-leucine; A, PTH-alanine; H, PTH-histidine; N, PTH-asparagine, respectively. Other abbreviations are same as in legend to Fig. 2.

applying this peptide to the protein sequencer. The result on the sequence of peak III is shown in Fig. 3. The sequence of peak III corresponded to I-A-G-E-A- S-R-L-A-H-^{83}Y-N-K, and phosphate was incorporated into ^{83}Y. Furthermore, peak V was eluted at the position of peak III after redigestion with lysylendopeptidase, and the molecular weight of peak V was estimated to be about 2800 by SDS-polyacrylamide gel electrophoresis (data not shown). These results indicated that peak V was the longer peptide consisting of 28 amino acids from ^{58}A to ^{85}K and the phosphate attached to ^{83}Y.

In the next experiment, peak IV was lyophilized and dissolved in H_2O and again applied to the reverse-phase C18 column on HPLC and was purified as described for peak III. The result on the peptide sequencing of peak IV was shown in Fig. 4. The sequence of peak IV corresponded to E-S-^{37}Y-S-V-^{40}Y-V-^{42}Y-K, and phosphate was incorporated into ^{40}Y. No phosphate was incorporated into ^{37}Y and ^{42}Y.

As the position of peak VI in reverse-phase HPLC column was identical with that of intact H2B histone (data not shown), peak VI seemed to be the intact undigested material. This corresponded to 2.8% of all of the phosphorylated H2B histone.

From the results mentioned above, the distribution of radioactive phosphate into ^{40}Y, ^{83}Y and ^{121}Y was calculated to be 0.138 mol (15.0%), 0.166 mol (18.0%) and

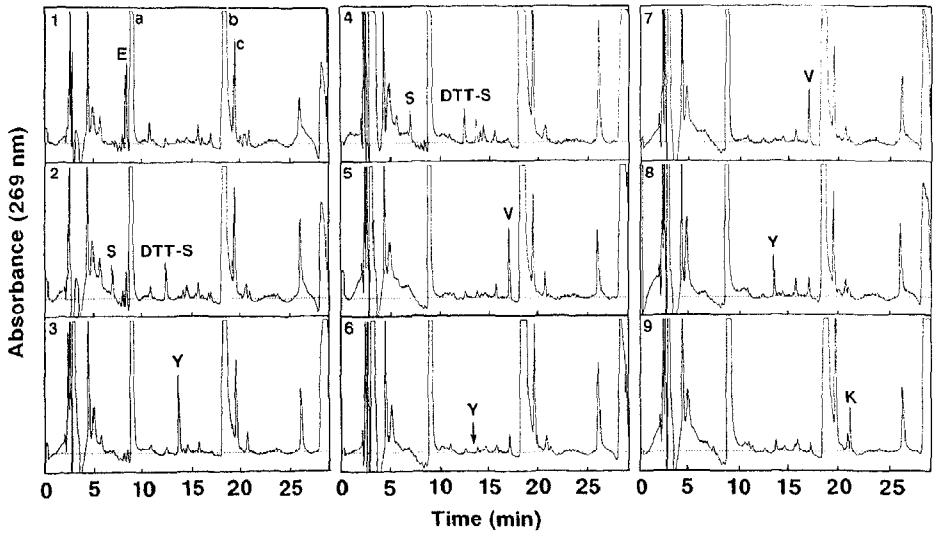

Fig. 4. Sequence analysis of peak IV after rechromatography by a reverse-phase column of HPLC. Phosphopeptide IV was redissolved in 100% acetonitrile and analyzed with the gas phase sequencer. The figure shows reverse-phase chromatograms of PTH derivatives corresponding to the first 9 cycles. *Abbreviations:* E, PTH-glutamic acid; V, PTH-valine, other abbreviations are same as legends to Figs. 2 and 3.

0.591 mol (64.2%), respectively.

4. DISCUSSION

Phosphorylation of H2B histone by protein-tyrosine kinases has been described by Stadtmauer and Rosen [10] using insulin receptor kinase as an enzyme. Graves et al. have reported that H2B histone phosphorylation by insulin receptor kinase is stimulated by the presence of Ca^{2+}-calmodulin [16]. However, the phosphorylation sites of H2B histone by insulin receptor kinase were not studied yet. We have recently reported that H2B histone is one of the best substrate proteins for 40 kDa kinase from porcine spleen [3] and its phosphorylation is inhibited by the presence of poly-basic amino acids [5,17]. However, the physiological significance of this tyrosine phosphorylation in H2B histone is still unknown as well as the case on the phosphorylation of serine residues by cAMP-dependent protein kinase, cGMP-dependent protein kinase [14] and protein kinase C [18].

In this paper we have clarified the phosphorylation sites of H2B histone by $p72^{syk}$. Three tyrosine residues in H2B histone are phosphorylated by this kinase (Fig. 5). ^{121}Y is identified as the major phosphorylated site and one glutamic acid is observed at 8 amino acids on the N-terminal side of this phosphorylated tyrosine. ^{83}Y and ^{40}Y are also shown to be phosphorylated. In these cases, one glutamic acid is also detected at 7 and 5 amino acids on the N-terminal side of the phosphorylated tyrosines, respectively. However, the location of each glutamic acid seems to be relatively far from the phosphorylated tyrosine. Previous analyses indicate that a cluster of acidic amino acids are usually detected closely near to the phosphorylated tyrosine [10,11]. In addition, it is also shown that ^{37}Y and ^{42}Y are not phosphorylated at all by the 40 kDa kinase in spite of the existence of one glutamic acid at 2 and 7 amino acids on the N-terminal side of each tyrosine residue, respectively. Based on these results, we would like to suppose that the single glutamic acid on the N-terminal side of each tyrosine residue does not seem to play an essential role for the recognition of H2B histone by this protein kinase. However, we could not completely rule out the possible role of these glutamic acids on the substrate recognition by this kinase. It is also shown that ^{40}Y is phosphorylated and this tyrosine is surrounded by valine residues. The sequence V-Y-V is also observed in [Val^5]angiotensin II which is one of the excellent substrates for many protein-tyrosine kinases in vitro. In conclusion we could not find out any consensus sequence for the recognition of this enzyme around the phosphorylated tyrosine residues in H2B histone.

^{26}KRKRSRKESYSVYVYKVLKQVHPN
IV
^{73}IAGEASRLAHYNKRSTITSR
III
^{112}SEGTKAVTKYTSSK
I

Fig. 5. Sequence around the phosphorylated tyrosine residues in H2B histone by 40 kDa kinase. Y with bold letter shows phosphotyrosine. Phosphopeptides of peak IV, III and I are underlined.

Tinker et al. suggested that β-turn in substrate proteins was important for the recognition by their kinase [13]. Geahlen and Harrison [9] also suggested the importance of higher-order structure of substrate proteins. By the analysis using Chou-Fasman program [19] we confirmed that secondary structures such as α-helix, β-sheet and/or β-turn were predicted to exist in the amino acid sequences surrounding these three phosphorylated tyrosine residues in H2B histone. However, it was also inferred from the same analysis that these secondary structures could be detected also around the sequences containing the unphosphorylated tyrosine residues (^{37}Y and ^{42}Y). Judging from these results, it seems to be hard to emphasize the importance of the secondary structure in substrate recognition by the 40 kDa kinase. In relation to this problem, it should also be pointed out that the method for the preparation of H2B histone using acid and organic solvent may seriously influence the higher-order structure originally existing in undenatured form of this histone molecule. At present more than 30 protein-tyrosine kinases have been reported, and it cannot be ruled out that each kinase may recognize a specific amino acid sequence in substrate protein as described previously for serine/threonine kinases [20]. Further studies seem to be necessary for understanding the mechanism of specific tyrosine phosphorylation by each protein-tyrosine kinase using various kinds of substrate proteins.

Acknowledgements: This investigation was supported by Grant-in-Aids for General Scientific Research and for Co-operative Research from the Ministry of Education, Science and Culture, Japan.

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