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Identification of the phosphoserine residue in histone H1 phosphorylated by protein kinase C

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The site-specific phosphorylation of bovine histone H1 by protein kinase C was investigated in order to further elucidate the substrate specificity of protein kinase C. Protein kinase C was found to phosphorylate histone H1 to 1 mol per mol. Using *N*-bromosuccinimide and thrombin digestions, the phosphorylation site was localized to the globular region of the protein, containing residues 71–122. A tryptic peptide containing the phosphorylation site was purified. Modification of the phosphoserine followed by amino acid sequence analysis demonstrated that protein kinase C phosphorylated histone H1 on serine 103. This sequence, Gly^{97}-Thr-Gly-Ala-Ser-Gly-Ser(PO_4)-Phe-Lys^{105}, supports the contention that basic amino acid residues C-terminal to the phosphorylation site are sufficient determinants for phosphorylation by protein kinase C.

Histone H1; Protein kinase C; Phosphorylation site

1. INTRODUCTION

The Ca^{2+}-phospholipid-dependent protein kinase, protein kinase C, phosphorylates many proteins in vitro and many potential in vivo substrates have been identified [1]. In addition many synthetic peptides have been examined in order to determine what sequences are recognized by this kinase. Although these studies have demonstrated a requirement for basic residues near the target serine residue, no single consensus sequence has emerged. For example some peptides have a basic residue on the N-terminal side of the phosphorylated serine [2–4] and other peptides have basic residues on the C-terminal side of the phosphorylated serine [5,6]. Some of the best peptide substrates that have been identified have basic residues on both sides of the phosphorylated serine [4,7]. Relatively less is known about the residues phosphorylated in proteins. For example histone H1 is a good substrate for this enzyme, but early findings [8,9] that protein kinase C and cAMP-dependent protein kinase phosphorylate the same site near the N-terminal (variously referred to as serine 35 to serine 38) were later contradicted [10]. Recently we determined that histone H1 phosphorylated by protein kinase C is a substrate which is quite specific for protein phosphatase 2A, whereas histone H1 phosphorylated by cAMP-dependent protein kinase is preferentially dephosphorylated by protein phosphatase 1 [11]. In the present study we used a recently developed method [12] to show that the residue phosphorylated by protein kinase C is serine 103. This phosphorylation site has a lysine residue on the C-terminal side of the phosphorylated residue but no basic residues on the N-terminal side and differs from the site phosphorylated by cAMP-dependent protein kinase.

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2. MATERIALS AND METHODS

2.1. *Materials*

Calf thymus histone H1 was prepared by the method of Sanders [13]. Protein kinase C was isolated from rat brain by the method of Sahyoun et al. [14]. The catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) was prepared as previously described [15]. *N*-Bromosuccinimide

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and thrombin (T-6634) were purchased from Sigma. Trypsin-TPCK was purchased from Worthington.

2.2. *Histone H1 phosphorylation*

Histone H1, phosphorylated by protein kinase C [H1(C)], was prepared by a modification [11] of the procedure of Kishimoto et al. [16]. In a total volume of 1 ml, 0.5 mg of histone H1 was incubated at 30°C in buffer (20 mM Tris-HCl, pH 7.50, at room temperature, 10 mM Mg^{2+}, 0.1 mM $CaCl_2$, 0.1 mM EGTA) containing 32 μg phosphatidylserine, 80 nM 12-*O*-tetradecanoylphorbol-13-acetate, 1 mM dithiothreitol, 1500 units protein kinase C, and 0.1 mM [γ-^{32}P]ATP, specific activity 200–500 cpm/pmol. The reaction was monitored, stopped and purified of contaminating proteins and ATP as in [11]. Histone H1 phosphorylated by protein kinase A [H1(A)], was prepared as above except that protein kinase A replaced protein kinase C and calcium, phospholipid and phorbol ester were eliminated from the reaction.

2.3. *Preparation of peptide fragments*

N-Bromosuccinimide cleavage [17] of histone H1(A) or H1(C) (100 nmol/ml) was carried out in 0.9 N acetic acid containing *N*-bromosuccinimide (1 μmol/ml) for 5 min at 25°C. The reaction mixture was lyophilized and then analyzed by SDS-polyacrylamide gel electrophoresis [18]. The digestion of histone H1(A) or H1(C) (150 nmol/ml) by thrombin [19] was performed in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, at room temperature, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 20 NIH units thrombin/ml for 40 min at 30°C. The reaction was terminated by addition to the SDS sample buffer [18] and the peptides analyzed by SDS-polyacrylamide gel electrophoresis.

2.4. *Preparation and purification of a phosphotryptic peptide*

A phosphotryptic peptide was prepared by incubating 43 nmol of H1(C) at 30°C in 1 ml containing 50 mM Tris-HCl, pH 7.5, 2 M urea and 24 μg trypsin. The reaction was terminated after 10 h by adding 9 μl of 5% trifluoroacetic acid (TFA). The peptide was purified by isocratic elution from a reversed-phase C-18 column (Synchropak RP-P) equilibrated with 0.1% TFA at a flow rate of 1 ml/min. The ^{32}P peak fractions which eluted between 10 and 13 min were pooled and then rechromatographed on a different reversed-phase C-18 column (Brownlee). The phosphopeptide was eluted from the second column with a 0–60% linear acetonitrile gradient in 0.08% TFA over 60 min. A single absorbance peak coinciding with the major radioactive peak (see fig.3) was collected, lyophilized and stored at −20°C.

2.5. *Amino acid composition and amino acid sequence*

Peptides were hydrolyzed in 6 N HCl at 110°C for 22 h. Amino acid composition was determined with a Beckman amino acid analyzer (model 6300) using ninhydrin chemistry. Amino acid sequence was determined with an Applied Biosystems 470 gas-phase sequencer. PTH-amino acid derivatives were analyzed on a C-18 reversed-phase column utilizing the Waters PTH-analysis program.

2.6. *Identification of phosphoserine*

To determine the site of phosphorylation, a procedure developed by Hastings and Reimann was employed [12]. Briefly, the phosphate of the tryptic peptide was removed by β-elimination. This was followed by the addition of pyridoxamine to the resulting dehydroalanine residue. Subsequent photolysis of the pyridoxamino adduct converted it to a form which could be identified after Edman degradation.

3. RESULTS AND DISCUSSION

Iwasa et al. [10] found protein kinase C was able to incorporate up to 2 mol of phosphate per mol of histone H1 on serine and threonine residues. We found, under the assay conditions presented here, protein kinase C phosphorylated histone H1 to 1 mol/mol on a serine residue only (fig.1). Addition of ATP and additional kinase after the ^{32}P reached a plateau did not increase the stoichiometry of phosphorylation. These results were not altered upon limited proteolysis of the protein kinase C to convert it to a Ca^{2+}-phospholipid-independent form (not shown). Digestion of proteins by *N*-bromosuccinimide is specific for cleavage at tryptophan, tyrosine and histidine residues [21], of which there is a single tyrosine at position 70 in bovine histone H1 [22]. Therefore the procedure yields a smaller amino-terminal peptide (residues 1–70) and a larger carboxyl-terminal peptide (residues 71–220). The autoradiograms in fig.2A of the SDS gels show that digestion of H1(A) produces a smaller labeled peptide than digestion of H1(C). Protein kinase A is known to phosphorylate the smaller, amino-terminal peptide [23], therefore the protein kinase C phosphorylation site must be on the larger carboxyl-terminal peptide. In addition to SDS gels, the phosphopeptides have been resolved on a HPLC TSK 2000 (LKB) with similar results (not shown). This supports the work of Iwasa et al. [10] localizing the site of protein kinase C phosphorylation to the carboxyl-terminal half of the protein.

Hartman et al. [24] have reported that the preferred site on histone H1 cleaved by thrombin is the sequence Pro-Lys-Pro-Lys^{122}-Lys-Ala-Gly, C-terminal to lysine 122. Histone H1 digested by thrombin yielded two major peptides which were readily separated by SDS gel electrophoresis or TSK 2000 chromatography (not shown). H1(A) and H1(C) digested with thrombin each produced a radioactive peptide that migrated to the same position on SDS gels (fig.2B) and on TSK 2000 chromatography (not shown). The labeled peptide migrated slightly faster than the larger peptide

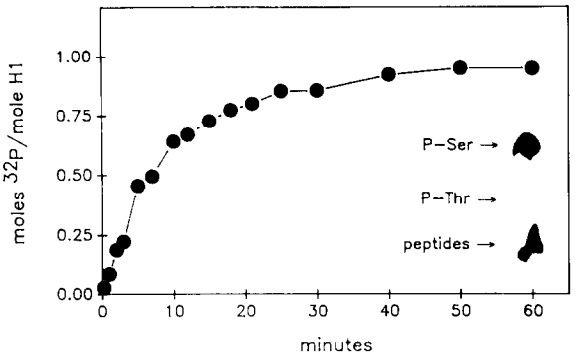

Fig.1. Phosphorylation of histone H1 by protein kinase C. ^{32}P incorporation into histone H1, catalyzed by protein kinase C, was measured by spotting at the indicated times. 2 μl of reaction on P-81 paper (Whatman), washing in 0.5% phosphoric acid, drying and counting in a liquid scintillation counter. Additional kinase and ATP were added at 40 and 50 min, respectively. (Inset) ^{32}P-histone H1 phosphorylated by protein kinase C for 60 min was hydrolyzed and analyzed for phosphoamino acid content [20].

generated by cleavage with *N*-bromosuccinimide. This size peptide is consistent with cleavage at lysine 122. Because the protein kinase A site and the protein kinase C site appear to be on the same peptide with thrombin digestion, this indicates that the protein kinase C phosphorylation site is amino-terminal to lysine 122. These results taken together further support the conclusion that the protein

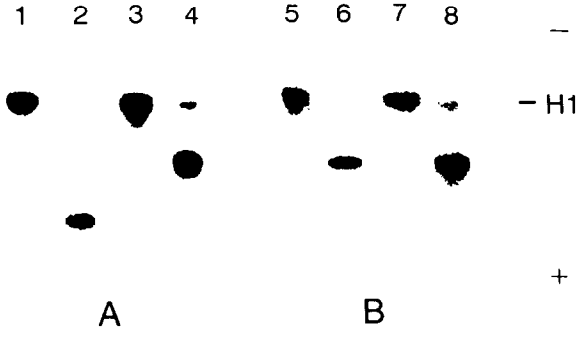

Fig.2. SDS gel electrophoretic analysis of H1(A) and H1(C) cleaved by *N*-bromosuccinimide or thrombin. H1(A) and H1(C) were electrophoresed on a 15% gel before and after *N*-bromosuccinimide (panel A) or thrombin cleavage (panel B). Autoradiographs of the following samples are presented (lanes): 1 and 2, H1(A) before and after *N*-bromosuccinimide treatment; 3 and 4, H1(C) before and after *N*-bromosuccinimide treatment; 5 and 6, H1(A), before and after thrombin treatment; 7 and 8, H1(C) before and after thrombin treatment.

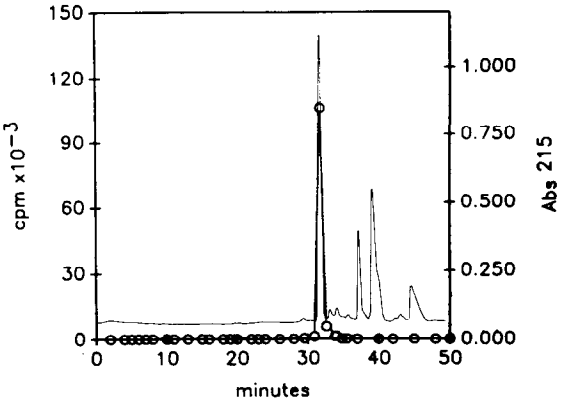

Fig.3. Purification of phosphopeptide by reversed-phase chromatography. The second C-18 column was developed as described in the text. (○) cpm as determined by Cherenkov counting; (——) absorbance at 215 nm.

kinase C phosphorylation site is confined to a region between tyrosine 70 and lysine 122 on a serine residue. This corresponds to the hydrophobic, globular region of the histone H1 protein [25].

To isolate a phosphopeptide for sequence analysis, H1(C) was extensively digested with trypsin and the phosphopeptide was purified by reversed-phase chromatography (fig.3). The composition and sequence of this phosphopeptide corresponded to bovine histone H1 97–105 (Gly-Thr-Gly-Ala-Ser-Gly-Ser-Phe-Lys) (not shown). Although sequence information for histone H1 is available only to residue 152 [26], the presence of phenylalanine and a high glycine content are unique to this peptide. This is a highly conserved sequence in histone H1 that is present in all

Table 1

Automated Edman degradation of modified peptide

Cycle	PTH-amino acid	pmol
1	Gly	2690
2	Thr	1410
3	Gly	1810
4	Ala	2970
5	Ser	370
6	Gly	1140
7	mod. Ser	1180
8	Phe	920
9	Lys	360

bovine, rabbit, pig, rat and trout subtypes that have been sequenced [22,26–29].

The presence of two serines in the phosphopeptide complicated identification of the phosphorylated residue, although the absence of an identifiable PTH derivative in the cycle corresponding to serine 103 in the sequence suggested that this was the phosphorylated residue [30]. To establish that serine 103 was the phosphorylated residue, the peptide was sequenced after modification as described in section 2 using a newly developed procedure for sequencing phosphopeptides [12]. The presence of a single modified residue at cycle 7 established that Ser 103 was the phosphorylated amino acid (table 1).

The selective phosphorylation of serine 103 by protein kinase C in the presence of a preferred kinase A site at serine 38 illustrates the differences in site specificity between these two enzymes. This is an excellent example of a good protein kinase C substrate containing the sequence Ser-Xaa-Lys with no basic amino acids N-terminal to the phosphorylation site. Also, phosphorylation of serine 103 by protein kinase C, supports the contention of Romhanyi et al. [31] that histone kinase II, which appears to phosphorylate serine 103 [32], is a proteolytic fragment of protein kinase C.

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