INVESTIGATIONS OF MAJOR SITES PHOSPHORYLATED IN HISTONE H1 BY KINASES FROM DIFFERENT STAGES OF THE CELL CYCLE

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1. Introduction

The cyclic nature of the process of H1 histone phosphorylation has been demonstrated in several reports [1–4] where the extent of phosphorylation was shown to change throughout the cell cycle, and the distribution of phosphorylation within the H1 histone was correlated with stages of the cell cycle in the sense that the amino-terminal region was compared to the carboxy-terminal region [5,6]. These experiments with in vivo phosphorylation produce quite complicated patterns of phosphopeptides [2,7] and more importantly, the phosphopeptides from synchronized cell culture are produced in very meager amounts. In order to identify the amino acid sequences of the phosphopeptides it is advantageous to carry out the phosphorylations in vitro. Langan [8] used this approach to identify some sites modified by the kinases of rapidly growing cells. The present work is an extension of this latter approach, but instead of using kinases generally associated with growth, it used kinases prepared from four different stages of the cell cycle for the in vitro phosphorylation of H1 histone. The major peptide sequences phosphorylated by these kinases were isolated and identified for correlation with the progression of the cell cycle.

2. Materials and methods

Protein kinases were purified by ammonium sulfate saturation of homogenized embryos which development was stopped at a particular stage of the cell cycle. The fractions containing the bulk of phosphokinase activity were precipitated between 35% and 65% of saturation, and this preparation was further fractionated by gel filtration on a Sephadex G-200 column (2.5 × 90 cm).

The phosphorylation of histone H1 or its large carboxy-terminal fragments (2 mg) by protein kinases samples (enzyme/histone ratio is about 1:100) was run for 12 h at 37°C in 2 ml reaction mixture containing 5×10^{-2} M Tris–HCl, pH 7.4; 1×10^{-2} M $MgCl_2$; 1×10^{-3} M dithiothreitol; 3×10^{-4} M EGTA and 3×10^{-4} M [γ-^{32}P]ATP.

The tryptic digest of the [^{32}P]phosphohistone H1 or its phosphorylated fragments (37°C, 16 h, trypsin: histone 1:100) was fractionated by finger-printing on Whatman 3MM paper, in the first direction, electrophoresis (80 V/cm) at pH 3.5 for 45 min, and in the second, chromatography in pyridine/butanol/acetic acid/water (10:15:3:12). The position of the radioactive peptide on the fingerprint was determined by radioautography using an RT-1 X-ray film (4 h exposure).

The amino acid composition of eluted [^{32}P]phosphopeptides was studied using a quantitative method of amino acid analysis [9] based upon their dansylation followed by two-dimensional thin-layer chromatography on polyamide plates. Spots of DNS-amino acids were scanned using a PMQ-II (Opton) spectro-

photometer with a 6 × 0.1 mm slit. All the peptides obtained were partially sequenced using Edman procedure, with the identification of amino-terminal amino acid residues in the form of their DNS-derivatives.

Carboxy-terminal amino acid residues were determined after hydrolysis of peptides with carboxypeptidase B and DNS-Cl treatment.

3. Results

The kinases studied were those that could be isolated from synchronous cultures of the sea urchin, *Strongylocentrotus intermedius*, by application of the procedure [10] previously used for the cAMP dependent kinase of pig brain except that DEAE cellulose chromatography was omitted. Enzymes representing G1, S, G2, and early mitotic stages were prepared from embryos 5, 20, 45 and 60 min after fertilization, respectively. As substrates for this series of enzymes, we used whole calf thymus H1 histone and two large carboxy-terminal fragments; one, representing residues 72–213, was obtained by cleavage with *N*-bromosuccinimide [11], and the other, representing residues 107–213, was produced by limited chymotryptic hydrolysis [12].

Tryptic hydrolysates of ^{32}P-labelled histone were resolved by peptide mapping and the radioactive peptides identified by autoradiography as shown in fig.1. When G1 kinase was used, 80% of the radioactivity was found in peptide A of fig.1a. When either S kinase or G2 kinase was used 90–95% of the radioactivity occurred in peptide A of fig.1b, and when mitotic kinase was used peptides A, B and C of fig.1c contained 70% of the radioactivity, (there was too little in peptide D for sequence analysis). These major peptides were eluted and analysed for terminal residues and amino acid composition. Dansyl Edman degradations gave partial sequences as shown in table 1. There are also listed in table 1 comparable sequences [13–16] from H1 histone of rabbit thymus with their appropriate residue numbers.

The comparison of the present sequences with those already established for rabbit thymus H1 histone allows us to propose the sequences shown, and their locations within the H1 histone molecule. When the large carboxy-terminal fragments were studied, the results were consistent with the locations proposed.

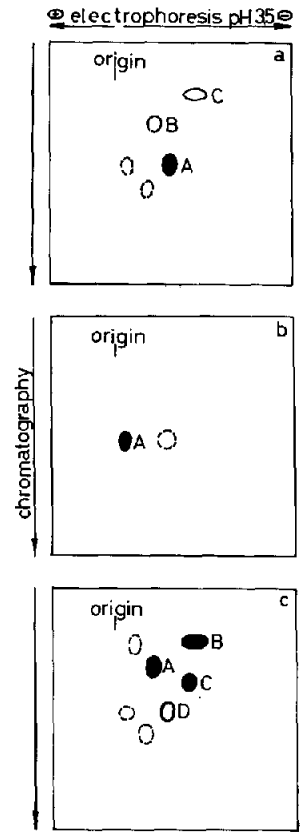

Fig.1. The autoradiographs of fingerprints of tryptic digest of H1 histone phosphorylated by (a) G1, (b) S or G2, and (c) early mitotic enzyme samples.

For example, only peptide A of fig.1b was found in both of the large fragments as well as in whole H1 histone.

A phosphorylated peptide [17,18] produced by the action of cAMP-dependent kinase was located [13] in the primary structure of H1 histone identifying Ser 37 as the phosphorylation site. That same site was phosphorylated in the present work by the G1 kinase. This phosphorylation is not dependent on the synthesis of DNA but might be involved in the regulation of some metabolic processes [18] or in the preparation of the chromatin for its replication. The kinases from stages S and G2 that phosphorylated mainly Ser 114, most likely do relate to DNA replication and the formation of newly synthesized chromatin fibrils.

Table 1
Structures of phosphorylated peptides

Stage of cell cycle	Peptide	Structure	Comparable sequence from a rabbit thymus H1 histone[a]
G1	A[b]	Lys (Ala, Ser, Gly, Pro, Pro, Val, Ser, Glu, Leu, Ile, Thr) Lys	37 Lys Lys Ala Ser Gly Pro Pro Val Ser Glu Leu Ile Thr Lys
S + G2	A	X Ala Ser (Gly, Glu, Ala) Lys	Lys Ala Ala Ser Gly Glu Ala Lys 114
M	A	Ser Pro Lys	Lys Ser Pro Lys 180
	B	Val Ala Lys (Ser, Pro) Lys	Lys Val Ala Lys Ser Pro Lys 180
			? (Lys Val Ala Lys Pro Lys Ser Pro Ala Lys) 173
	C	Thr Pro Lys	Thr Pro Lys 153

[a] Refs [13–16]
[b] Sequence determined as the peptide phosphorylated by cAMP-dependent kinase [17,18]

Phosphorylation by the kinase from early mitosis presented the most complicated picture. One of the peptides found, Ser–Pro–Lys was probably derived from one of the others to simplify the picture. However, while our analysis of peptide B in fig.1c indicates a sequence corresponding to residues 177–182, it must be noted that even a heavy contamination of that sequence by the one from residues 168–175 is difficult to rule out rigorously considering the precision of the methods at hand. In work to be reported later [20] the kinase from mitosis was resolved on Sephadex G-200 to yield a fraction that phosphorylated mainly Ser-180 and another enzyme fraction that modified Thr-153.

Although sites 153 and 180 have been reported [8], he did not observe the 114 site that we did, while we failed to detect the phosphorylation at Thr-136 which he found. This discrepancy may be due to a difference in the source of enzymes used, but it may also illustrate a limitation to this way of correlating phosphorylation sites with cell cycle events. The partial purification of the kinases might have excluded certain activities, and while this is an advantage for simplifying the work on peptide structure, it must be kept in mind that the present analysis is not a comprehensive one. Another possible limitation to this approach is that some aspects of chromatin structure other than the H1 molecule per se, could affect phosphorylation patterns, but these would be overlooked. Nevertheless, our results give a clear demonstration of the multiplicity of H1 histone kinases and the diversity of their actions, suggesting a number of specific manipulations of H1 histone in the dynamics of chromatin structures.

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