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PHOSPHORYLATION OF CALF THYMUS H1 HISTONE BY CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE*

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SUMMARY: Ca^{2+}-activated, phospholipid-dependent protein kinase recently found in mammalian tissues (Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695) is able to phosphorylate five fractions of calf thymus histone. H1 histone serves as a preferential substrate, and approximately two moles of phosphate are incorporated into every mole of this histone. Analysis on the N-bromosuccinimide-bisected fragments of this radioactive histone has revealed that the enzyme phosphorylates preferentially seryl and threonyl residues located in the carboxyl-terminal half of this histone molecule.

Structural modification of histone may be intimately related to the organization of chromatin (1). Among such modification reactions phosphorylation of H1 histone has been proposed to be one of the possible candidates which regulate chromosomal condensation (2-6). It seems to be well established that this histone is phosphorylated at Ser-38 through cyclic AMP-dependent processes in in vitro as well as in vivo systems (7,8). Cyclic GMP-dependent protein kinase also preferentially phosphorylates Ser-38 (9). Another line of evidence has shown that in late G1 through M phase multiple phosphorylation may occur at both seryl and threonyl residues in the carboxyl-terminal half of this histone molecule (10-12). Although the existence of

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specific protein kinases has been repeatedly implied (5,10,13), neither the properties nor the regulatory processes of such enzymes that are responsible for the growth-associated phosphorylation have yet been substantiated. It has been generally accepted that Ca^{2+} is indispensable for cell proliferation and that various extracellular stimulators including mitogenic substances evoke rapid turnover of phosphatidylinositol in target cell membranes (for reviews see Refs. 14-16). In preceding reports from this laboratory (17,18), a new multifunctional protein kinase has been described that requires the simultaneous presence of Ca^{2+} and phospholipid. The activation of this enzyme appears to be directly related to phosphatidylinositol turnover, since a trace amount of unsaturated diacylglycerol which is derived from phosphatidylinositol hydrolysis markedly increases the affinity of enzyme for Ca^{2+} as well as for phospholipid, and thereby serves as a second messenger for initiating this protein kinase activation (19,20). In this communication we show that this unique protein kinase actively phosphorylates H1 histone, particularly the seryl and threonyl residues located at the carboxyl-terminal half and thus seems to have a potential to participate in transmembrane control of the growth-associated H1 histone phosphorylation mentioned above. Ca^{2+}-activated, phospholipid-dependent protein kinase and cyclic AMP-dependent protein kinase will be referred to as protein kinases C and A, respectively.

EXPERIMENTAL PROCEDURES

Protein kinase C was partially purified from rat brain soluble fraction as previously described (21). Protein kinase A was prepared from rabbit skeletal muscle as described earlier (22). These enzyme preparations were free of each other and of endogenous phosphate acceptor proteins. Histone fractions were prepared from calf thymus and purified as described (9). Protein kinase C was routinely assayed with H1 histone as phosphate acceptor in the presence of Ca^{2+}, phospholipid, and diolein. The detailed conditions are given in Table I. A phospholipid mixture employed for this study was prepared from human erythrocyte ghosts as described previously (20). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (24), and other reagents were obtained from commercial sources.

For analysis of the phosphorylated sites, radioactive H1 histone was bisected chemically at tyrosine residue (Tyr-74) by the N-bromosuccinimide procedure, and the resulting two peptide fragments were separated by gel filtration on a Sephadex G-100 column as described by Sherod et al. (25). Acid hydrolysis of radioactive histone fractions, and analysis of phosphoserine and phosphothreonine were carried out as described previously (26). Tryptic peptides and fingerprints were prepared under the conditions specified earlier (23).

RESULTS AND DISCUSSION

Both protein kinases C and A utilized histone as phosphate acceptor. Table I shows the relative reaction velocities for five histone fractions. Protein kinase C reacted most rapidly with H1 histone, and after prolonged incubation approximately two moles of phosphate was incorporated into every mole of this histone. Under similar conditions protein kinase A greatly favored H2B histone, and

Table I

Relative rates of phosphorylation of histone fractions by protein kinases C and A

Protein kinase C was assayed in the reaction mixture (0.25 ml) which contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium acetate, 50 nmol of $CaCl_2$, 2.5 nmol of [γ-^{32}P]ATP (1 x 10^5 cpm/nmol), 10 μg of phospholipid, 1 μg of diolein, 0.4 μg of protein kinase C, and 50 μg each of histone fractions as indicated. Phospholipid was first mixed with diolein in a small volume of chloroform. After the chloroform was removed in vacuo, the mixture was suspended in 20 mM Tris/HCl at pH 7.5 by sonication as described previously (20) and employed for the assay. The incubation was carried out for 3 min at 30°C. The reactions were stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). The radioactivity was determined as described earlier (23). Protein kinase A was assayed in the same reaction mixture except that, instead of $CaCl_2$, phospholipid, and diolein, 250 pmol of cyclic AMP was added. Protein kinase A (0.5 μg of protein) was employed.

Histone fraction	Acid-precipitable radioactivity	
	Protein kinase C	Protein kinase A
	(cpm)	(cpm)
H1 histone	17,480	3,830
H2A histone	2,850	2,470
H2B histone	4,380	17,000
H3 histone	1,210	468
H4 histone	240	260

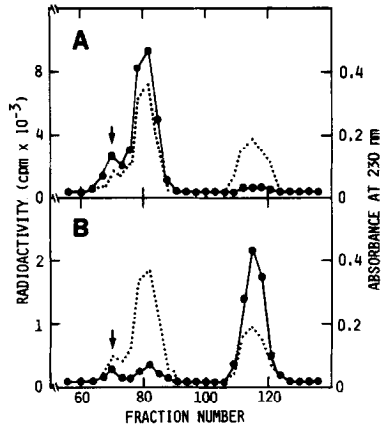

Fig. 1. Separation of N-bromosuccinimide fragments of H1 histone phosphorylated by protein kinases C and A. H1 histone (500 µg of protein) was incubated for 120 min in a large-scale reaction mixture (10 times the standard assay) with an excess amount of protein kinase and radioactive ATP; with 100 µg of protein kinase C, 200 µg of protein kinase A, and with 250 nmol of [γ-^{32}P]ATP (2 x 10^8 cpm). Under these conditions no additional incorporation of ^{32}P was observed at the end of incubation unless more histone was supplemented to the reaction mixture. Each radioactive H1 histone was extracted from the reaction mixture with 1.5 mg of non-radioactive H1 histone as a carrier protein as described previously (9), and was subjected to treatment with N-bromosuccinimide. The bisected fragments were separated by gel filtration on a Sephadex G-100 column (135 x 1.1 cm). A, with protein kinase C; and B, with protein kinase A. (●——●), radioactivity; and (·····), absorbance at 230 nm. Arrows indicate non-bisected histone.

the reaction with H1 histone was relatively slow. After prolonged incubation about a half mole of phosphate was incorporated per mole of H1 histone. The reason of this poor stoichiometry was unknown, but was attributed to, at least in part, the microheterogeneity of this histone fraction (27).[1/]

The experiment given in Fig. 1 was designed to show that protein kinases C and A preferred each different portion of this histone molecule. H1 histone was phosphorylated for prolonged period of time, and bisected chemically at Tyr-74 with N-bromosuccinimide. Then, the resulting two polypeptide fragments were separated by a Sephadex G-100 column. Approximate molecular weight for the amino-terminal fragment was 6,000, and that for carboxyl-terminal fragment was 15,000.

1/ This H1 histone preparation contained practically no detectable phosphate before being phosphorylated.

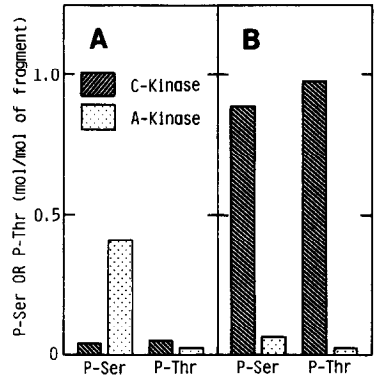

Fig. 2. Phosphoserine and phosphothreonine produced from N-bromosuccinimide fragments of H1 histone phosphorylated by protein kinases C and A. The amino- and carboxyl-terminal fragments of H1 histone phosphorylated by protein kinases C and A were prepared as described in Fig. 1. Each radioactive fragment was subjected to acid hydrolysis and determination of phosphoserine and phosphothreonine were made as described previously (23,26); under the conditions employed the recovery of ^{32}P in phosphoserine and phosphothreonine was about 35% of the initial total radioactivity. The amount of each phosphoamino acid was estimated from the ratio of phosphoserine to phosphothreonine. A, amino-terminal fragment; and B, carboxyl-terminal fragment. C-kinase and A-kinase indicate protein kinases C and A, respectively. P-Ser and P-Thr indicate phosphoserine and phosphothreonine, respectively.

The small peak shown with an arrow was non-bisected histone.[2/] The results clearly indicate that protein kinase C greatly favored the carboxyl-terminal half of this histone, although the enzyme also could phosphorylate the amino-terminal half very slowly. Inversely, protein kinase A reacted more rapidly with the amino-terminal half. Acid hydrolysis of the radioactive peptide fragments revealed that both protein kinases C and A phosphorylated seryl and threonyl residues in the carboxyl-terminal half, although the reaction by protein kinase A was extremely slow.[3/] Protein kinase A reacted rapidly with the seryl residue located in the amino-terminal half. This seryl residue has been previously identified as Ser-38 (8,9). These re-

2/ This peak is not an intact molecule of H1 histone, but a product of an abnormal N-bromosuccinimide cleavage reaction as described by Sherod et al. (25).

3/ The seryl and threonyl residues in the carboxyl-terminal half are not identified, since the primary sequence of calf thymus H1 histone has not been clarified. Apparently, both protein kinases C and A recognize the same specific seryl and threonyl residues as suggested by fingerprint analysis of each radioactive peptide fragment, although the relative reaction velocities towards these residues are markedly different. The precise specificity and mechanism of substrate recognition of these multifunctional protein kinases may not be established until these specific seryl and threonyl residues are all identified.

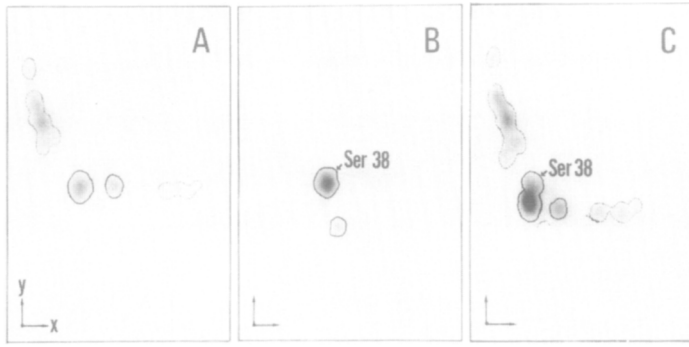

Fig. 3. Autoradiography of tryptic phosphopeptides of radioactive H1 histone phosphorylated by protein kinases C and A. Radioactive H1 histone phosphorylated by protein kinases C and A were prepared as described in Fig. 1. Each radioactive H1 histone preparation was digested with trypsin and subjected to paper chromatography (direction x) followed by paper electrophoresis (direction y) under the conditions as described previously (23) except that the solvent system for paper chromatography was n-butanol/acetic acid/water (3/1/1). A, with protein kinase C; B, with protein kinase A; and C, cochromatography.

sults are quantitatively summarized in Fig. 2. Fingerprint analysis of the radioactive whole H1 histone molecule gave apparently different patterns for these enzymes as shown in Fig. 3. The major radioactive spot obtained for protein kinase A has been previously identified as a phosphopeptide containing Ser-38 (9).

It may be noted that various hormones and other extracellular messengers provoke rapid turnover of phosphatidylinositol in many types of target tissues. However, several of the receptors which have been accepted to produce cyclic AMP do not induce such phosphatidylinositol response (16). Thus, it is likely that protein kinases C and A play roles in transferring distinctly different information across the cell membrane, and eventually lead to the phosphorylation of specific sites in the H1 histone molecule. Nevertheless, the precise mechanism of such transmembrane signalling for histone phosphorylation has remained unknown, since no information has been available as to whether phosphatidylinositol turnover takes place in nuclear membranes upon stimulation by extracellular messengers. It is theoretically possible that Ca^{2+} influx directly activates

protein kinase C at nuclear membranes (17-20) or that the proteolytically activated enzyme moves into the nucleus. As described earlier (21,28), protein kinase C may be alternatively activated by limited proteolysis by a Ca^{2+}-dependent protease and the catalytic fragment thus produced is fully active without Ca^{2+} and membrane phospholipid.

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