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THIOPHOSPHORYLATION AND PHOSPHORYLATION OF CHROMATIN PROTEINS FROM CALF THYMUS IN VITRO

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Thiophosphorylation and phosphorylation of 5 % perchloric acid extractable proteins from calf thymus chromatin were studied using a cyclic GMP-dependent protein kinase from bovine lung and a nuclear protein kinase II from rat liver. The phosphorylation reaction catalyzed by nuclear protein kinase II utilized [γ-^{35}S]ATP as a phosphate donor almost as efficiently as [γ-^{32}P]ATP, but the cGMP-dependent protein kinase mediated phosphorylation by [^{35}S]ATP was about 20 times less effective than that by [^{32}P]ATP. In addition, using [^{35}S]ATP instead of [^{32}P]ATP changed markedly the cGMP-dependent phosphorylation pattern of the PCA-extractable proteins as examined by gel electrophoresis. Thus, depending on the type of protein kinase, the results from thiophosphorylation and phosphorylation reactions may vary considerably.

The thiophosphate analogue of ATP, adenosine 5'-O-(3-thiotriphosphate), was first synthesized by Goody and Eckstein (1). In 1980, Sun et al. (2) introduced a method that uses a radioactive thiophosphate derivative of ATP to detect newly phosphorylated protein molecules. The thiophosphate analogue of ATP has been employed as a thiophosphate group donor in various kinase-mediated transfer reactions, e.g., in the activation of phosphorylase b and phosphorylase kinase in rabbit muscle (3) and in the phosphorylation of myosin light chains (4). In addition, this ATP analogue is the donor for phosphorylation of histones H1, H2B, and H3 and high mobility group protein HMG 1 in vitro (2). In cultures of growing cells, nuclear proteins have also been shown to undergo thiophosphorylation (5).

Protein kinases are grouped into at least two types of activities: cyclic nucleotide dependent (e.g. cGMP-dependent) and cyclic nucleotide independent (e.g. nuclear protein kinase II) (6, 7). Cyclic nucleotide dependent kinases are active only with ATP as phosphate donor, whereas cyclic nucleotide independent nuclear protein kinase II (casein kinase II) utilizes GTP almost as well as ATP.

The high mobility group proteins, which include HMG 1, HMG 2, HMG 14 and HMG 17, constitute a subclass of non-histone chromosomal proteins that occur widely throughout the

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eukaryotic kingdom. These proteins can be selectively extracted from cell material using 5 % perchloric acid, which also extracts histone H1 (8). Of the four main types of HMG proteins, HMG 14 and HMG 17 (as well as histone H1) have been shown to be phosphorylated by both cyclic nucleotide dependent and cyclic nucleotide independent protein kinases (9, 10). In this study thiophosphorylation and phosphorylation of the perchloric acid extractable chromatin proteins from calf thymus were compared using cGMP-dependent protein kinase and nuclear protein kinase II as phosphotransferases.

EXPERIMENTAL PROCEDURE

Adult male and female rats (Wistar BD-IX) were obtained from the Experimental Animal Center of the University of Kuopio and fed ad libitum with a standard diet. Calf thymus and bovine lung were obtained fresh from a local slaughterhouse. [γ-^{32}P]ATP (3 Ci/mmol) and [γ-^{35}S]ATP (50 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. NCS solubilizer was obtained from Amersham/Searle Co. Reagents for polyacrylamide gel electrophoresis, the cellulose thin-layer sheets for high-voltage electrophoresis and the X-Omat AR-films for autoradiography were from Eastman Kodak Co.

The perchloric acid extract of calf thymus was prepared by extracting the tissue with 5 % PCA as described by Sanders et al. (11). Histone H1 was removed in part by fractional acetone precipitation (8). HMG 14 from calf thymus was purified by CM-Sephadex C-25 chromatography as described earlier (10). Histone H1 was isolated from calf thymus using the method of Johns et al. (12). Nuclear protein kinase II from rat liver and cGMP-dependent protein kinase from bovine lung were purified as described previously (9, 10).

The standard mixture for the phosphorylation assay with cGMP-dependent protein kinase contained in a total volume of 0.1 ml: 0.1-0.2 mg of the substrate protein, 50 mM glycerol phosphate (pH 7.5), 0.3 mM EGTA, 2 mM theophylline, 10 mM $MgCl_2$, 2 µM cGMP, and indicated amounts of the protein kinase and radioactive nucleoside triphosphate. The phosphorylation assay with nuclear protein kinase II contained in a final volume of 0.1 ml: 0.2 mg of the substrate protein, 40 mM Tris-HCl (pH 7.2), 6.25 mM $MgCl_2$ and indicated amounts of the enzyme and [^{35}S]ATP or [^{32}P]ATP. The reaction was initiated by the addition of the enzyme and carried out at 30°C for 90 min. The reaction was terminated and the samples were analyzed as described in the legend to Fig. 1, or aliquots of the samples (30 µg protein) were subjected to acid polyacrylamide gel electrophoresis in the presence of 4 M urea (9) as described in Fig. 2.

To identify the phosphoamino acids, phosphorylated HMG 14 and histone H1 were purified as described earlier (13) and hydrolyzed in 6 M HCl for 2 hours at 100°C. Aliquots of 25 µl were mixed with 0.1 µmol each of the phosphoserine and phosphothreonine standards and subjected to thin-layer high-voltage electrophoresis for 60 min as described in the legend to Fig. 3.

RESULTS AND DISCUSSION

The time curves for phosphorylation and thiophosphorylation of the PCA-extractable proteins from calf thymus are shown in Fig. 1. Total phosphorylation of the extract proteins by cGMP-dependent protein kinase was decreased from 22 pmoles to 0.8 pmoles when [^{32}P]ATP was substituted for [^{35}S]ATP. No decrease was observed in reactions catalyzed by nuclear protein kinase II (3-5 pmoles of radioactive nucleotide incorporated). Thus, in

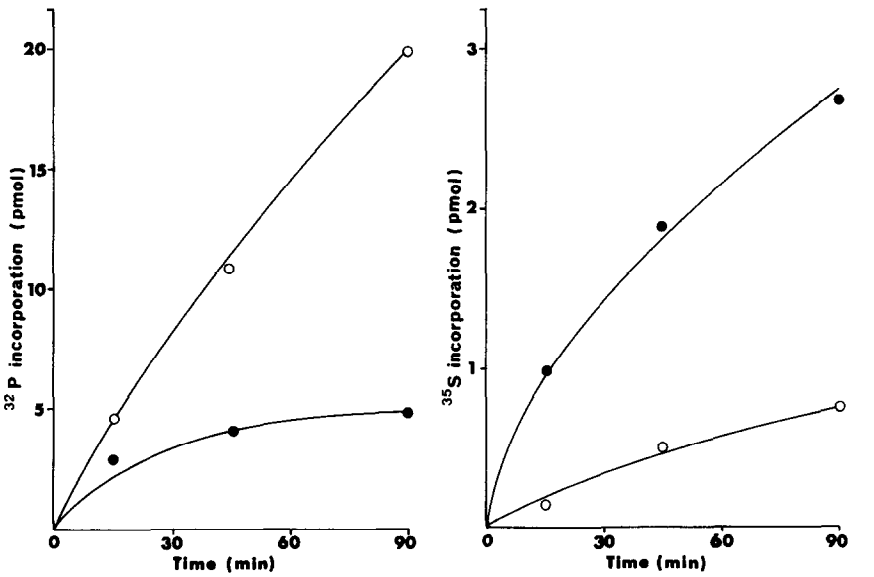

Figure 1. Phosphorylation of calf thymus perchloric acid (PCA) extractable proteins by cGMP-dependent protein kinase (o) or nuclear protein kinase II (●) in the presence of [^{32}P]ATP (A) and [^{35}S]ATP (B). In the standard assay mixture, 0.1 mg of the extract was phosphorylated by 4.0 μg of cGMP-dependent kinase or by 1.5 μg of nuclear protein kinase II in the presence of 24 μM [^{32}P]ATP or 6 μM [^{35}S]ATP. After incubation at 30°C for 90 min, the mixture was chilled to 0°C and the proteins were precipitated by addition of 3 ml of cold 30 % trichloroacetic acid (TCA). The sample was mixed with Vortex mixer, and the precipitate was collected on a glass fiber filter (Whatman GF/A, 2.4 cm), which was prewashed with 20 % TCA, dried and counted for radioactivity in 5 ml of toluene-based counting fluid.

contrast to cGMP-dependent protein kinase, nuclear protein kinase II utilizes [^{35}S]ATP as a phosphate donor almost as efficiently as it uses [^{32}P]ATP.

The cGMP-dependent protein kinase catalyzes phosphorylation of three prominent protein bands (Fig. 2A). On the basis of mobility on acetic acid/urea-slab gels and autoradiography, these proteins were identified as histone H1, a proteolytic fragment of histone H1 (so-called HMG 8) (14) and HMG 14. In contrast to the cGMP-dependent protein kinase, nuclear protein kinase II did not appreciably phosphorylate HMG proteins or H1. When [^{35}S]ATP was used as a phosphate donor instead of [^{32}P]ATP, the phosphorylation pattern changed markedly (Fig. 2 B). Nuclear protein kinase II phosphorylated four unidentified proteins of higher molecular weight, which were present in small amounts in the chromatin extract. In thiophosphorylation mediated by cGMP-dependent kinase, ^{35}S was transferred to the same three proteins as ^{32}P . The relative phosphorylation of histone H1, however, decreased from 34 to 18 % and that of HMG 14 from 33 to 9 %. When

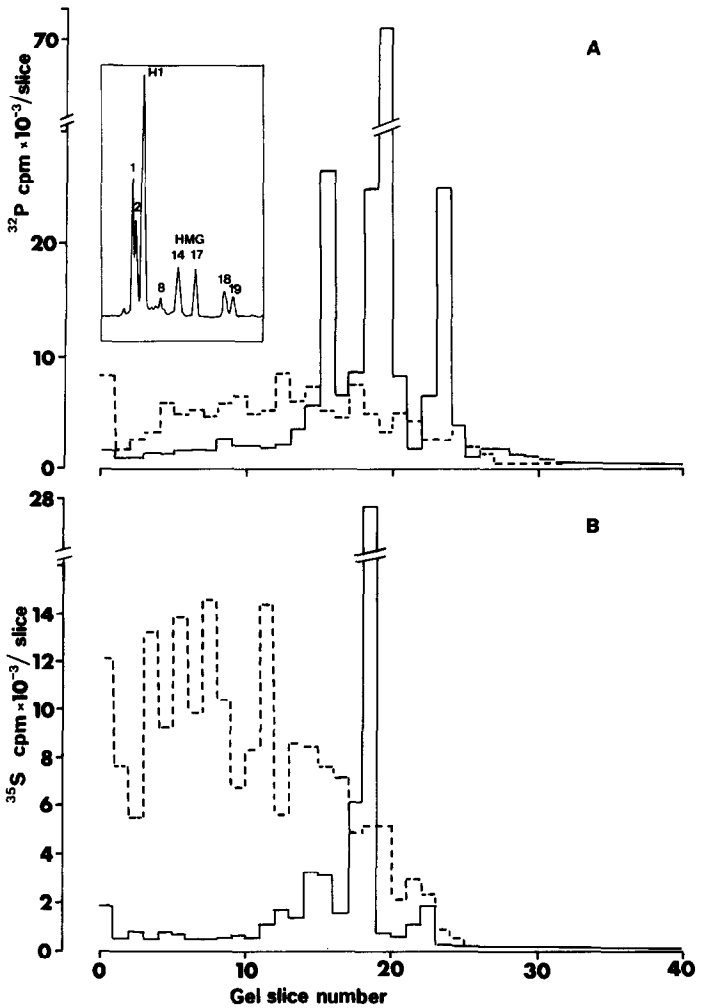

Figure 2. Analysis of the phosphorylated proteins from calf thymus PCA-extract by acid-urea polyacrylamide gel electrophoresis in 15 % cylindrical gels. In the standard mixture, 0.1 mg of the extract protein was phosphorylated separately by cGMP-dependent kinase (solid line) and nuclear protein kinase II (dotted line) as in Fig. 1 in the presence of [^{32}P] ATP (A) or [^{35}S]ATP (B). After electrophoresis, the gels were cut, and 2 mm slices were placed in separate counting vials and incubated with 50 µl of water and 0.45 ml of NCS solubilizer at 37°C overnight. The slices were then counted for radioactivity with 5 ml of toluene-based counting fluid. After electrophoresis parallel gels were stained with 0.2 % Procion blue in 7 % acetic acid, and destained with 40 % ethanol at 55°C. The densitogram of the stained protein bands is shown in the insert.

control incubations without added substrate proteins were analyzed using the same gel system, we found no significant incorporation of radioactivity. The results thus indicate that when [^{32}P]ATP is replaced by [^{35}S]ATP as a phosphate donor, the total phosphorylation of the 5 % PCA-extractable proteins from calf thymus chromatin by cGMP-dependent protein kinase but not by nuclear protein kinase II decreases significantly. In addition, the

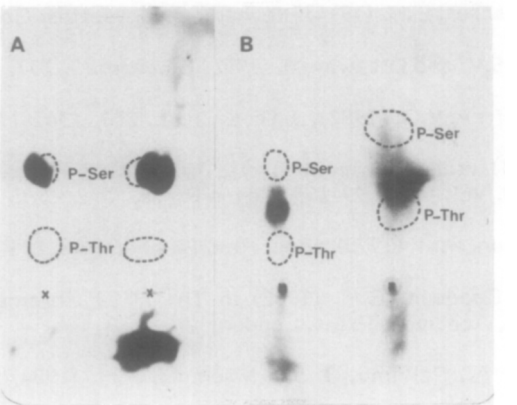

Figure 3. Analysis of the phosphorylated (A) and thiophosphorylated (B) amino acid residues in purified HMG 14 and histone H1 by thin-layer high-voltage electrophoresis and autoradiography. In the standard assay mixture, 1 mg of purified HMG 14 or H1 was phosphorylated separately by cGMP-dependent protein kinase or nuclear protein kinase II as in Fig. 1. The phosphorylated proteins were hydrolyzed in 6M HCl for 2 h at 100°C. The hydrolysates were then analyzed by thin-layer high-voltage electrophoresis at pH 1.9 (2200 V, 60 min) on cellulose sheets, stained with 0.2 % ninhydrin, and subjected to autoradiography.

relative proportion of different protein substrates phosphorylated by cGMP-dependent protein kinase changes markedly when [^{32}P]ATP is replaced by [^{35}S]ATP.

Thiophosphate has been reported to be incorporated into both serine and threonine residues of histone H1 in growing Hela cells (2). Using thin-layer high-voltage electrophoresis and autoradiography, we analyzed the phosphorylated and thiophosphorylated amino acid residues from the hydrolysates of purified H1 and HMG 14. As shown in Fig. 3 A, only one major radioactive band, which comigrated with the authentic phosphoserine standard, was detected in the presence of [^{32}P] ATP. The radioactivity derived from thiophosphorylation did not comigrate with either of the phosphoprotein standards but rather between phosphoserine and phosphothreonine (Fig. 3 B). The thiophosphate group differs somewhat in character and structure from phosphate and this may affect the migration of [^{35}S] phosphoserine (15).

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